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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07B 61/00 // C09B 69/10, 11/08, C07H 21/02, 21/04, 1/00, C07K 17/08, 17/02

A1

(11) International Publication Number: WO 00/32542

(43) International Publication Date: 8 June 2000 (08.06.00)

(21) International Application Number: PCT/AU99/01065

(22) International Filing Date: 30 November 1999 (30.11.99)

(30) Priority Data:
PP 7372 30 November 1998 (30.11.98) AU

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CARRIERS FOR COMBINATORIAL COMPOUND LIBRARIES

(57) Abstract

A carrier pre-encoded with information sufficient to distinguish it from a heterogeneous population of carriers is disclosed on which a compound can be synthesised. The carrier has two attributes integrally associated therewith, which attributes are detectable and/or quantifiable during synthesis of the compound and which define a code identifying the carrier before, during and after said synthesis, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier. The invention also encompasses a plurality of carriers that are pre-encoded as above and a method of synthesising and deconvoluting a combinatorial library using such carriers.

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"CARRIERS FOR COMBINATORIAL COMPOUND LIBRARIES"

FIELD OF THE INVENTION

THIS INVENTION relates generally to combinatorial compound libraries. In particular, the present invention relates to carriers having distinctive codes for use in combinatorial compound synthesis as well as to combinatorial compound libraries produced with those carriers. The invention is also concerned with a novel method for structural deconvolution of a combinatorial library member.

BACKGROUND OF THE INVENTION

Recently, there has been substantial interest in devising facile combinatorial technologies to synthesise molecular libraries of immense diversity. A major utility of such libraries is that they can be screened for various biological, pharmacological or chemical activities in the pursuit of novel lead compounds.

In essence, combinatorial technologies are predicated on systematic assembly of a collection of chemical building blocks or synthons in many combinations using chemical, biological or biosynthetic procedures. The potential number "N" of different individual library members produced by such an assembly can be calculated as a function of the number of different synthons available for each step "b" and the number of synthetic steps in the reaction scheme "x", according to the following formula: $N = b^x$. Thus, a library of nonapeptides constructed using 20 different amino acids (*i.e.*, the synthons) could include as many as 20^9 or 5.1×10^{11} different library members.

Combinatorial libraries may be assembled by a number of methods including the "split-process-recombine" or "split synthesis" method described first by Furka *et al.* (1988, *14th Int. Congr. Biochem., Prague, Czechoslovakia* **5**: 47; 1991, *Int. J. Pept. Protein Res.* **37**: 487-493) and Lam *et al.* (1991, *Nature* 354:82-84), and reviewed later by Eichler *et al.* (1995, Medicinal Research Reviews 15(6): 481-496) and Balkenhohl *et al.* (1996, *Angew. Chem. Int. Ed. Engl.* **35**: 2288-2337). The split synthesis method involves dividing a plurality of solid supports such as polymer beads into n equal fractions representative of the number of available synthons for each step of the synthesis (e.g., 20 L-amino acids, 4 different nucleotides etc), coupling a single respective synthon to each polymer bead of a

corresponding fraction, and then thoroughly mixing the polymer beads of all the fractions together. This process is repeated for a total of x cycles to produce a stochastic collection of up to N^x different compounds. Thus, by employing syntheses where the coupling involves the addition of synthons such as amino acids, nucleotides, sugars, lipids or heterocyclic compounds, where the synthons may be naturally-occurring, synthetic or combinations thereof, one may create a large number of molecularly diverse compounds.

The molecular libraries so produced can then be screened for the identification of novel ligands that interact with a receptor target of interest. For any given receptor target, the probability of successfully identifying a potent ligand through a process of randomly screening molecular repertoires will undoubtedly increase as the size and structural diversity of the library is also increased. However, an inherent difficulty of producing large libraries of this type is the ability to determine the reaction history of any chosen combinatorial library member to thereby deconvolute its structure. For large numbers of solid supports and large numbers of steps and/or processing methods, this "deconvolution" procedure is particularly difficult. In many practical cases, where high throughput and fast analysis is required, this problem is intractable by conventional methods.

The conventional split synthesis technologies referred to above present difficulties when it is desired to detect and isolate a combinatorial library member of interest. In this regard, it is necessary to first cleave the member from its solid support before identifying the member by techniques such as mass spectroscopy or HPLC. This is time consuming and cumbersome and in some cases, cleavage is not possible.

A number of groups have attempted to overcome these prior art deficiencies by use of chemical encoding which relies on reactions different and orthogonal from those used in the synthesis of the combinatorial library member. For example, Janda (1994, *Proc. Natl. Acad. Sci. USA* **91**: 10779-10785) describes a method in which each synthesis step of a combinatorial library member is followed by an independent coupling of an identifier tag to a solid support. Through a series of sequential chemical steps, a sequence of identifier tags are built up in parallel with the compounds being synthesised on the solid support. When the combinatorial synthesis is complete, the sequence of operations any particular solid support has gone through may be retraced by separately analysing the tag sequence. Accordingly, use of identifier tags in this manner provides a means whereby

one can identify which synthon reaction an individual solid support has experienced in the synthesis of a combinatorial library member. The identifier tag also records the step in the synthesis series in which the solid support visited that synthon reaction. In this regard, reference may be made to International Publication WO93/06121 in which Dower *et al.* disclose a general stochastic method for synthesising a combinatorial compound library on solid supports from which library members may be cleaved to provide a soluble library. The identifier tag may be attached directly to a member of the library with or without an accompanying particle, to a linker attached to the member, to the solid support on which the member is synthesised or to a second particle attached to the member-carrying particle.

However, while Dower *et al.* (*supra*) refer very broadly to the types of identifier tags that may be utilised in combinatorial library formation, the only experimental evidence that they provide is the use of oligonucleotides as tags which are identifiable by sequencing or hybridisation. They also make reference to amplifying the oligonucleotide tag by PCR if only trace amounts of oligonucleotide are available for analysis. However, it will be appreciated that such identification methods are time consuming and inefficient. For example, use of PCR may result in PCR product contamination making it necessary to introduce further measures to overcome this problem as described by Dower *et al.* (*supra*). It is also necessary to sequence amplified DNA and this involves an additional step in the identification procedure.

In U.S. Patent No. 5,721,099, Still *et al.* disclose a process of constructing complex combinatorial chemical libraries of compounds wherein each compound is produced by a single reaction series and is bound to an individual solid support on which is bound a combination of four distinguishable identifiers which differ from one another. The combination provides a specific formula comprising a tag component capable of analysis and a linking component capable of being selectively cleaved to release the tag component. Each identifier or combination thereof encodes information at a particular stage in the reaction series for the compound bound to the solid support. The identifiers are used in combination with one another to form a binary or higher order encoding system permitting a relatively small number of identifiers to be used to encode a relatively large number of reaction products. However, the method of Still *et al.* (*supra*) does not provide for direct identification of the tag component on the solid support. In this regard, it is essential prior to analysis of a combinatorial library that each tag component be cleaved

from the support thus creating at least one additional step which is time consuming and inefficient. Accordingly, the same disadvantages relevant to the method of Dower *et al.* also apply to that of Still *et al.*

In addition to the disadvantages mentioned above, chemical encoding techniques such as those described by Junda (1994, *supra*), Dower *et al.* (*supra*) and Still *et al.* (*supra*) rely on parallel, orthogonal synthesis of identifier tags which adds substantially to the time taken for completion of a combinatorial synthesis and has the potential to interfere with the synthesis.

Spectrometric encoding methods have also been described in which decoding of a library member is permitted by placing a solid support directly into a spectrometer for analysis. This eliminates the need for a chemical cleavage step. For example, Geysen *et al.* (1996, *Chem. Biol.* 3: 679-688) describe a method in which isotopically varied tags are used to encode a reaction history. A mass spectrometer is used to decode the reaction history by measuring the ratiometric signal afforded by the multiply isotopically labelled tags. A disadvantage of this method is the relatively small number of multiply isotopically labeled reagents that are commercially available.

Optical encoding techniques have also been described in which a solid support's absorption or fluorescence emission spectrum is measured. For example, reference may be made to Sebestyén *et al.* (1993, *Pept. 1992 Proc. 22nd Eur. Pept. Symp.* 63-64), Campian *et al.* (1994, In *Innovation and Perspectives on Solid Phase Synthesis* Epton, R., Birmingham: Mayflower, 469-472) and Egner *et al.* (1997, *Chem. Commun.* 735-736) who describe the use of both chromophoric and/or fluorescent tags for bead labeling in peptide combinatorial synthesis. Although this use provides advantage for deconvoluting a library member's structure by simply reading a bead's absorption or fluorescence emission spectrum, the encoding of a large library would require the use of many chromophores or fluorophores where spectral superimposition would be a likely drawback.

Yamashita and Weinstock (International Publication WO 95/32425) disclose the coupling on beads of (i) fluorescently labelled tags having intensities that differ by a factor of at least 2, and/or (ii) multiple different fluorescent tags that can be used in varying ratios, to encode a combinatorial library. Such beads may be used in concert with flow cytometry to construct a series of combinatorial libraries by split synthesis procedure. In

this regard, a first combinatorial library is prepared by conducting a specified set of reaction sequences on tagged beads according to (i) and (ii) to encode each choice of synthon in the first stage of combinatorial synthesis (the term "stage" corresponds to a step of a sequential synthesis of a combinatorial library member). A second combinatorial library is prepared from substantially the same specified set of reaction sequences as the first library wherein the tagged beads are combined and separated prior to the first reaction sequence and the beads are sorted prior to the second reaction sequence to encode each choice of synthon in the second stage. The sorting step is characterised in that the beads are sorted into groups of similarly tagged beads. Additional libraries are prepared according to the preparation of the second library except that the sort step is performed prior to a different stage in the combinatorial synthesis. The number of libraries constructed in the series will therefore equal to the total number of stages in the combinatorial synthesis wherein a different stage is encoded in each library. After synthesis is complete, each library is tested for biological activity and a population analysis analogous to Structure Activity Relationship (SAR) studies is conducted for each library to reveal which variable synthon(s) are important for activity and which are not. Although this method has advantages in relation to providing a lead structure, it is necessary to construct and analyse multiple libraries commensurate with the number of stages used for the combinatorial synthesis, which is cumbersome and time consuming.

Kaye and Tracey (International Publication WO 97/15390) describe a physical encoding system in which chemically inert solid particles are each labelled with a unique machine readable code. The code may be a binary code although higher codes and alphanumerics are contemplated. The code may consist of surface deformations including pits, holes, hollows, grooves or notches or any combination of these. Such deformations are applied by micromachining. Alternatively, the code may reside in the shape of the particle itself. Solid particles comprising a first phase for combinatorial synthesis and a second phase containing a machine readable code are exemplified wherein the second phase may be superimposed on, or encapsulated within, the first phase. The microscopic code on the particles may be interrogated and read using a microscope-based image capture and processing system. The encoding system of Kaye and Tracey provides advantage in that the machine readable code may be read "on-the-fly" between process steps of a combinatorial synthesis thus allowing the process sequence, or audit trail, for

each bead to be recorded. However, this system suffers from a number of drawbacks in that specialised purpose-built machinery is required for producing the solid particles and for reading the code. For example, the application of code deformations onto the solid particles requires expensive micromachining technology, computer aided design (CAD) tools for designing the required particle geometry, as well as manufacture of appropriate photolithographic masks for delineating the particle shapes. In addition, there is a need to utilise specialised image processing systems and software for observing a particle from several different directions to accurately determine and verify a given code.

Many of the disadvantages of the known methods described above as well as many of the needs not met by them are addressed by the present invention, which, as described more fully hereinafter, provides numerous advantages over the above-described methods.

SUMMARY OF THE INVENTION

According to one aspect of the invention, there is provided a carrier on which a compound can be synthesised, wherein said carrier has at least two attributes integrally associated therewith, which attributes are detectable and/or quantifiable during synthesis of the compound and which define a code identifying the carrier before, during and after said synthesis, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier.

Preferably, at least one of said attributes is comprised within or internally of the carrier.

Suitably, at least one of said attributes is an electromagnetic radiation-related attribute.

Preferably, the electromagnetic radiation-related attribute is selected from the group consisting of fluorescence emission, luminescence, phosphorescence, infrared radiation, electromagnetic scattering including light and X-ray scattering, light transmittance, light absorbance and electrical impedance.

Preferably, the electromagnetic radiation-related attribute is a light emitting, light transmitting or light absorbing attribute detectable by illuminating the carrier with incident light of one or more selected wavelengths or of one or more selected vectors.

In another aspect, the invention provides a plurality of carriers on which a plurality of different compounds can be synthesised, including a population of detectably distinct carriers each having a code, which distinctively identifies a respective carrier before, during and after said synthesis from other carriers, and which is characterised by at least two detectable and/or quantifiable attributes integrally associated with the carrier, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier.

In yet another aspect, the invention resides in a method of producing a plurality of carriers including a population having detectably distinct carriers, comprising the steps of:

-

(a) preparing a plurality of carriers having different codes wherein each code is characterised by at least two detectable and/or quantifiable attributes integrally associated with a respective carrier;

(b) detecting and/or quantifying the said attributes of each carrier to thereby assign a code for each carrier;

(c) identifying carriers having distinctive codes;

(d) identifying carriers having similar codes; and

(e) sorting the carriers having distinctive codes from the carriers having non-distinctive codes to thereby provide a plurality of carriers including a population having detectably distinct codes.

In yet another aspect, the invention resides in a plurality of carriers having detectably distinct codes resulting from the method as broadly described above.

In a further aspect, the invention provides a method of synthesising and deconvoluting a combinatorial library comprising the steps of: -

(a) apportioning in a stochastic manner among a plurality of reaction vessels a plurality of carriers on which a plurality of different compounds can be synthesised, wherein said plurality of carriers includes a population of detectably distinct carriers each having a code, which distinctively identifies a respective carrier before, during and after said synthesis from other carriers, and which is characterised by at least two detectable and/or quantifiable attributes integrally associated with the carrier, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier;

(b) determining and recording the codes of said plurality of carriers in order to track the movement of individual detectably distinct carriers into particular reaction vessels of said plurality of reaction vessels, wherein said codes are determined prior to step (d);

(c) reacting the carriers in each reaction vessel with a synthon;

(d) pooling the carriers from each reaction vessel;

(e) apportioning the carriers in a stochastic manner among the plurality of reaction vessels;

(f) reacting the carriers in each reaction vessel with another synthon;

(g) recording the codes of said plurality of carriers in order to track the movement of individual detectably distinct carriers into particular reaction vessels of said plurality of reaction vessels, wherein said codes are recorded after step (e) or step (f);

(h) pooling the carriers from each reaction vessel;

(i) iterating steps (e) through (h) as necessary to create a combinatorial compound library wherein member compounds of the library are associated with the detectably distinct carriers and wherein codes of the detectably distinct carriers are deconvolutable using tracking data provided by said recordal steps to identify the sequence of reactions experienced by the said detectably distinct carriers.

The invention in yet a further aspect refers to a combinatorial compound library produced by the aforementioned method.

The invention in a still further aspect resides in a kit comprising: -

(a) a combinatorial compound library including a plurality of different compounds wherein each compound is attached to at least one of a plurality of carriers, which includes a population of detectably distinct carriers each having a distinctive code, which distinctively identifies a respective carrier before, during and after synthesis of a corresponding compound from other carriers, and which is characterised by at least two detectable and/or quantifiable attributes integrally associated with the carrier, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier; and

(b) tracking data on each distinctive code to identify the sequence of reactions experienced by a respective detectably distinct carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of a modern flow cytometer. The core (sample) stream is hydrodynamically focused before intercepting the laser beam at the observation point. MilliQ™ water was used as sheath fluid in the present investigation. The laser beam, core stream and optical array are mutually orthogonal at the observation point. A beam stop is placed before the FS detector to remove transmitted light.

Figure 2 is a schematic representation of one step in a split-process-recombine procedure, e.g. as discussed in the prior art in relation to the synthesis of peptide libraries.

Figure 3 is a schematic representation of the entire iterative split-process-recombine procedure referred to in Figure 1.

Figure 4 is a schematic representation of a division of two-dimensional parameter space into gridspaces. Note that the width of each gridspace can be different for each parameter.

Figure 5 is an example of a real-time algorithm for selecting optically unique microspheres. In panel (a), five microspheres have already been collected and hence the corresponding gridspace labels have been labelled full. In panel (b), a new microsphere

occupies a vacant gridspace and hence is sorted in panel (c). In panel (d), another new microsphere occupies a full gridspace and hence is rejected from the system in panel (e).

Figure 6 is a schematic representation of a refined method of selecting optically unique microspheres. Only microspheres that occupy the internal sort region are collected. No microspheres are collected from the buffer region.

Figure 7 is a reaction scheme for the coupling of isothiocyanates to primary amines.

Figure 8 shows fluorescence micrographs of (a) FITC-coated 2.5 μm microspheres (S1) and (b) QFITC-coated 2.5 μm microspheres (S2). Both micrographs are after six centrifugation-redispersion cycles using a U-MWB filter. Doublets and triplets are present from the original commercial synthesis.

Figure 9 show scanning electron micrographs of: (a) uncoated 2.5 μm microspheres, (b) FITC-coated 2.5 μm microspheres (S1), (c) uncoated 4 μm blue-greenF microspheres, and (d) QFITC-coated 4 μm blue-greenF microspheres (R7).

Figure 10 is a graph depicting Calibration of flow cytometer using Flow-Check™ microspheres. Each diluted sample (total volume 1 mL) was run for 2.00 minutes on MED flow rate (35 ± 5 μL min^{-1}). Calculated concentration of microspheres is 1.03 x 10^6 microspheres mL^{-1}.

Figure 11 is a graph showing three distinct populations in a mixture of 10 μm greenF, 10 μm redF and 12 μm red-greenF microspheres

Figure 12 is a graph showing a polygonal gate to collect 10 μm greenF population only. 100000 microspheres collected in 50-mL sheath fluid (MilliQ™ water).

Figure 13 is a graph showing a polygonal gate to collect 12 μm red-greenF population only. 100000 microspheres collected in 50-mL sheath fluid (MilliQ™ water).

Figure 14 is a fluorescence micrograph of an original mixture of three different microspheres. Green, red and orange (red-green) microspheres are distinguishable and well dispersed.

Figure 15 is a graphical representation of a mixture of fluorescently coated samples S1 (FITC), S2 (QFITC) and the non-fluorescent uncoated 2.5 μm microspheres. The ratio of red fluorescence to green fluorescence is fixed at low concentrations for a given fluorophore, hence the correlation within samples.

Figure 16 shows histograms of (a) FL1 values for non-fluorescent (black) and S1 (green). 25^{th} -75^{th} percentiles are 21-30 and 438-877 channel numbers for non-fluorescent and S1 respectively, and (b) FL3 values for non-fluorescent (black) and S2 (red). 25^{th} -75^{th} percentiles are 7-47 and 73-162 channel numbers for non-fluorescent and S2 respectively.

Figure 17 is a bivariate plot of FL1 and FL3 for uncoated 4 μm blue-greenF microspheres and three different concentrations of QFITC-coated microspheres (R1, R8, R9). Four micrometer blue-redF microspheres are included to represent QFITC-coated microspheres containing no green fluorescence. This mixture of microspheres is approaching optical diversity.

Figure 18 is a graph of increase in red fluorescence intensity with increasing amount of QFITC-APS added. The linearity of the graph suggests FRET is not occurring at these concentrations.

Figure 19 shows bivariate plots of the well-defined sorting gates and subsequent re-analysis for the two precision experiments (refer also Table E and Figure 23) using Flow-Check™ microspheres. The aggregate populations were caused by the <1000 rpm centrifugation required to concentrate the sorted microspheres from 50 mL to 0.5 mL. The size of the population recovered from the initial 200000 sorted microspheres is 10-20 %.

Figure 20 is an example of a frequency histogram used to determine *rl* for FL3. Note the effect that turbulence in the flow system has on the value of *rl*. By discarding the first 5000 events recorded, the turbulent data was removed. Greater than 99.9 % of the remaining single microspheres had a deviation less than 47 channel numbers from the lowest channel number in the initial sort (ie. 423), hence $rl = 47$, whereas before the turbulent data was removed, $rl > 75$.

Figure 21 is a graph showing the relationship between processing time and population size for the post-acquisition algorithm.

Figure 22 is a graph showing the theoretical limit for maximum number of unique microspheres (Ep = 50).

Figure 23 is a graph showing the relationship between processing time and number of iterations for the real-time algorithm.

Figure 24 is a graph showing the time for one iteration of the real-time algorithm for a given number of parameters.

Figure 25 is a graph showing the number of unique microspheres obtained as a function of population size for random data. The total number of available gridspaces is 10000.

Figure 26 is a graphical plot of an *optodiverse* population of QFITC-coated 4 μm blue-greenF microspheres on two parameters (FL1 and FL3) before pre-encoding.

Figure 27 is a schematic representation of the gridspaces of 56 optically unique microspheres extracted from population in Figure 29 (rl = rh = 30).

Figure 28 is a graph showing that the optimum value of w_i for U is 110 using conditions described above.

Figure 29 is a graph showing the prediction of the number of optically unique microspheres that can be extracted after a given period of time using Equation 5.4.

Figure 30 shows reproducibility of the mean values of the scattering/fluorescence signals of fluorescent silica particles. Seven bivariate plots are shown corresponding to seven passes through a flow cytometer of identical aliquots of the same sample of microspheres.

Figure 31 shows non-fluorescent 10.2 μm microspheres collected and repassed through a flow cytometer give reproducible scattering values. Two bivariate plots are shown of a well-defined sorting gate and subsequent reanalysis of the gated non-fluorescent microspheres.

Figure 32 shows non-fluorescent 10.2 and 21 μm microsphere mixtures collected and repassed through a flow cytometer give reproducible scattering values. Four bivariate

plots are shown of well-defined sorting gates and subsequent reanalysis of the gated mixtures of non-fluorescent microspheres.

Figure 33 shows fluorescent green polystyrene microspheres collected and repassed through the flow cytometer give reproducible scattering and fluorescence values. Four bivariate plots are shown of well-defined sorting gates and subsequent reanalysis of the gated fluorescent green microspheres.

Figure 34 shows non-fluorescent polystyrene/divinylbenzene (DVB) microspheres swelled in DMF for 3 hours and returned to Milli-Q water give scattering values similar to those that have not been subjected to DMF. Two bivariate plots of gated fluorescent polystyrene microspheres are illustrated, one population representing a control (Panel A) and the other representing microspheres exposed to DMF treatment (Panel B).

Figure 35 shows fluorescent red silica microspheres swelled in DMF for 3 hours and returned to Milli-Q water give scattering and fluorescence values similar to those that have not been subjected to DMF. Two bivariate plots of gated fluorescent silica microspheres are shown, one population representing a control (Panel A) and the other representing microspheres exposed to DMF treatment (Panel B).

Figure 36 shows two bivariate plots of gated fluorescent Tentagel microspheres, one population representing a control (Panel A) and the other representing microspheres having one amino acid coupled thereto (Panel B).

Figure 37 shows four bivariate plots of gated fluorescent silica microspheres, one population (Panel A, red fluorescence and side scatter; Panel C, red fluorescence and forward scatter) being collected, having a glycine coupled thereto and subsequently passed through a flow cytometer (Panel B, red fluorescence and side scatter; Panel D, red fluorescence and forward scatter).

Figure 38 shows three bivariate plots of gated fluorescent green (Panel A), fluorescent orange (Panel B) and fluorescent red (Panel C), 10.2 μm polyelectrolyte coated microspheres.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

The term "*carrier*" as used herein embraces a solid support with appropriate sites for compound synthesis and, in some embodiments, tag attachment. The carrier may have any suitable size or shape or composition. Preferably, carriers are heterogeneous in size, shape, or composition. In general, the carrier size is in the range of between about 1 nm to 1 mm. The carrier may be shaped in the form of spheres, cubes, rectangular prisms, pyramids, cones, ovoids, sheets or cylinders.

The term "*compound*" as used herein refers to molecules comprising a sequence of synthons, which includes any structural unit that can be formed and/or assembled by known or conceivable synthetic operations. Thus, the compounds of the present invention are formed from the chemical or enzymatic addition of synthons. Such compounds include, for example, both linear, cyclic, and branched oligomers or polymers of nucleic acids, polysaccharides, phospholipids, and peptides having, for example, either α-, β-, or ω-amino acids, heteropolymers in which, for example, a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulphides, polysiloxanes, polyimides, polyacetates, or other polymers which will be readily apparent to one skilled in the art upon review of this disclosure. The number quoted and the types of compounds listed are merely illustrative and are not limiting.

By "*features integrally associated with the carrier*" or "*features integrally associated therewith*" is meant features of the carrier and/or features of one or more elements, molecules, groups, tags and the like attached to the carrier.

By "*marker*" is meant any molecule or groups of molecules having one or more recognisable attribute including, but not restricted to, shape, size, colour, optical density, differential absorbance or emission of light, chemical reactivity, magnetic or electronic encoded information, or any other distinguishable attribute.

As used herein "*synthon*" includes any member of a set of molecules which can be joined together to form a desired compound. For example, synthons may include amino acids, carbonates, sulphones, sulfoxides, nucleosides, carbohydrates, ureas, phosphonates,

lipids, and esters. Alternatively, the synthons may comprise inorganic units such as for example silicates and aluminosilicates. Accordingly, a set of synthons useful in the present invention includes, but is not restricted to, for the example of peptide synthesis, the set of L-amino acids, D-amino acids, or synthetic amino acids. It will also be understood that different basis sets of synthons may be used at successive steps in the synthesis of a compound of the invention.

Throughout this specification and the appendant claims, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

2. *Carriers of the invention*

The present invention resides, at least in part, in a carrier on which a compound can be synthesised, wherein the carrier has at least two attributes integrally associated therewith, which attributes are detectable and/or quantifiable during synthesis of the compound. The attributes define a code identifying the carrier before, during and after synthesis of a compound, with the proviso that one of the attributes is other than shape, or surface deformation(s) of the carrier. Through the use of its plurality of detectable and/or quantifiable attributes, preferably optically detectable and/or quantifiable attributes, the carrier of the present invention provides more "pre-encoded" information compared to other carriers of the prior art and thus provides larger combinational library sizes that can be encoded. This "pre-encoded" information may be read by conventional flow cytometers and can be used to track the synthetic history of an individual carrier in a combinatorial process as described hereinafter. The present inventors have found that the larger the diversity of detectable and/or quantifiable attributes of a carrier, the greater the degree of decipherability or resolution of the carrier in a large population of carriers. In this regard, each detectable and/or quantifiable attribute of a carrier provides at least a part of the information required to distinctively identify the carrier. The larger the number of such attributes, the more detailed the identifying information that is compilable for a given carrier, which may be used to distinguish that carrier from other carriers.

The invention also encompasses a plurality of carriers including a population that are pre-encoded as above. Accordingly, each carrier of that population has a code, which

distinctively identifies a respective carrier before, during and after said synthesis from other carriers, and which is characterised by at least two detectable and/or quantifiable attributes integrally associated with the carrier, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier. The diversity of the said population of carriers, therefore, resides in carriers of said population having relative to each other different combinations of detectable attributes, which are used to provide distinctive codes for each of those carriers.

The carriers of the invention may be used in many applications, such as combinatorial chemistry procedures that do not involve a split-process-recombine procedure. Preferably, however, such assemblies are used in combinatorial chemistries, which involve a split-process-recombine procedure.

The carriers may comprise any solid material capable of providing a base for combinatorial synthesis. For example, the carriers may be polymeric supports such as polymeric beads, which are preferably formed from polystyrene cross-linked with 1-5% divinylbenzene. Polymeric beads may also be formed from hexamethylenediamine-polyacryl resins and related polymers, poly[N-{2-(4-hydroxylphenyl)ethyl}] acrylamide (*i.e.*, (one Q)), silica, cellulose beads, polystyrene beads poly(halomethylstyrene) beads, poly(halostyrene) beads, poly(acetoxystyrene) beads, latex beads, grafted copolymer beads such as polyethylene glycol/polystyrene, porous silicates for example controlled pore-glass beads, polyacrylamide beads for example poly(acryloylsarcosine methyl ester) beads, dimethylacrylamide beads optionally cross-linked with N,N'-bis-acrylolyl ethylene diamine, glass particles coated with a hydrophobic polymer inclusive of cross-linked polystyrene or a fluorinated ethylene polymer which provides a material having a rigid or semi-rigid surface, poly(N-acryloylpyrrolidine) resins, Wang™ resins, Pam resins, Merrifield™ resins, PAP and SPARE polyamide resins, polyethylene functionalised with acrylic acid, kieselguhr/polyamide (Pepsyn K), polyHipe™, PS/polydimethylacrylamide copolymers, CPG, PS macrobeads and Tentagel™, PEG-PS/DVB copolymers.

It will also be appreciated that the polymeric beads may be replaced by other suitable supports such as pins or chips as is known in the art, e.g. as discussed in Gordon *et al.* (1994, J. Med. Chem. 37(10):1385-1401). The beads may also comprise pellets, discs, capillaries, hollow fibres or needles as is known in the art. Reference also may be made to

International Publication WO93/06121, incorporated herein by reference, which describes a broad range of supports that may constitute carriers for use in present invention. By way of example, these carriers may be formed from appropriate materials inclusive of latex, glass, gold or other colloidal metal particles and the like. Reference may also be made to International Publications WO95/25737 or WO97/15390, incorporated herein by reference, which disclose examples of suitable carriers.

A plurality of carriers according to the invention may be prepared by any suitable method. Preferably, when colloidal particles including polymeric and ceramic particles are used as carriers, the colloid dispersion of such carriers is stabilised. Exemplary methods imparting colloidal stabilisation are described for example in Hunter, R. J. (1986, "Foundation of Colloid Science", Oxford University Press, Melbourne) and Napper, D.H. (1983, "Polymeric stabilisation of Colloidal Dispersions" Academic Press, London), the disclosures of which are incorporated herein by reference. In this regard, the most widely exploited effect of nonionic polymers on colloid stability is steric stabilisation, in which stability is imparted by polymer molecules that are absorbed onto, or attached to, the surface of the colloid particles. Persons of skill in the art will recognise that it is possible to impart stability by combinations of different stabilisation mechanisms: *e.g.*, surface charge on the particles can impact colloidal stability via electrostatic stabilisation, and an attached polyelectrolyte can impart stability by a combination of electrostatic and steric mechanisms (*electrosteric stabilisation*). Polymer that is in free solution can also influence colloid stability. Stabilisation by free polymer is well-documented (Napper 1983, *supra*) and is called *depletion stabilisation.*

Preferably, steric stabilisation of colloid dispersions is employed. In this regard, steric stabilisation is widely exploited because it offers several distinct advantages over electrostatic stabilisation. For example, one advantage is that aqueous sterically stabilised dispersions are comparatively insensitive to the presence of electrolytes because the dimensions of non-ionic chains vary relatively little with the electrolyte concentration. This contrasts sharply with the spatial extensions of electrical double layers, which are strongly dependent upon the ionic strength. It is apparent that at ionic strengths greater than *ca.* 10^{-2} mol dm^{-3}, electrical double layer thicknesses have shrunk to such an extent that the electrostatic repulsion may no longer outweigh the van der Waals attraction. This accounts for the coagulation of electrostatically stabilised dispersions on the addition of

electrolyte. Another advantage is that it is equally effective in both aqueous and non-aqueous dispersion media. This contrasts with electrostatic stabilisation, which is relatively ineffective in non-polar dispersion media. In addition, steric stabilisation is equally effective at both high and low volume fractions of the dispersed phase; the high volume fraction dispersions displaying relatively low viscosities. Other advantages of sterically stabilised dispersions include good freeze-thaw stability, which can be a desirable attribute in some applications, and the ability to be flocculated reversibly, which is less common with electrostatically stabilised dispersions.

Any suitable stabilising moiety may be used for stabilising colloidal dispersions. Exemplary stabilising moieties that impact on colloidal stability are given in Table A.

TABLE A

Classification of some sterically stabilised dispersions at room temperature and pressure

Stabilising Moieties	Dispersion medium			Type of Stabilisation
	Type	Example	Flocculation	
Poly(oxyethlene)	aqueous	0.39 M $MgSO_4$	heating to UCFT	enthalpic
Poly(vinyl alcohol)	aqueous	2 M NaCl	heating to UCFT	enthalpic
Poly(acrylic acid	aqueous	0.2 M HCl	cooling to LCFT	entropic
Poly(acrylamide)	aqueous	2.1 M $(NH_4)_2SO_4$	cooling to LCFT	entropic
Polystyrene	non-aqueous	cyclopentane	cooling to LCFT	entropic
Poly(iso-butylene)	non-aqueous	2-methylbutane	heating to UCFT	enthalpic

A significant advance of the present invention over the prior art is the provision of a carrier with a combination of at least two detectable and/or quantifiable attributes with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier. The said attributes characterise a code that permits facile deconvolution of a plurality of reaction steps experienced by the carrier by methods as described, for example, hereinafter. In a preferred embodiment, at least one of said attributes is comprised within or internally of the carrier. This reduces exposure of the attribute to solvents required for compound synthesis on the carrier and thus, the *encoded information* corresponding to the attribute is more stable providing for greater reproducibility of the code.

It is preferred that at least one of the attributes of a carrier is an electromagnetic radiation-related attribute suitably selected from the group consisting of atomic or molecular fluorescence emission, luminescence, phosphorescence, infrared radiation, electromagnetic scattering including light and X-ray scattering, light transmittance, light absorbance and electrical impedance.

The fluorescence emission may result from excitation of one or more fluorescent markers attached to, or contained within, the carrier. In the case of two or more fluorescent markers being utilised, the markers may be the same wherein the markers contain varying amounts of a fluorophore and are therefore intensity-differentiated. Alternatively, the markers may be different wherein they are present in a ratio of 1:1 or varying ratios. Reference may be made in this regard to Yamashita *et al.* (International Publication WO 95/32425) which is incorporated herein by reference.

Exemplary fluorophores which may be used in accordance with the present invention include those discussed by Dower *et al.* (International Publication WO 93/06121 which is incorporated by reference herein). Preferably, fluorescent dyes are employed. Any suitable fluorescent dye may be used for incorporation into the carrier of the invention. For example, reference may be made to U.S. Patents 5,573,909 (Singer *et al.*, which is incorporated herein by reference) and 5,326,692 (Brinkley *et al.*, which is incorporated herein by reference) which describe a plethora of fluorescent dyes. Reference may also be made to fluorescent dyes described in U.S. Patent Nos. 5,227,487, 5,274,113, 5,405,975, 5,433,896, 5,442,045, 5,451,663, 5,453,517, 5,459,276, 5,516,864, 5,648,270 and 5,723,218 which are all incorporated herein by reference.

One or more of the fluorescent dyes are preferably incorporated into a microparticle, such as a polymeric microparticle or ceramic microparticle. Such microparticles may be attached to the carrier by use of colloidal interactions as for example disclosed by Trau and Bryant in copending International Application PCT/AU98/00944, incorporated herein by reference. Preferably, the fluorescent polymeric or ceramic microparticle comprises the carrier for combinatorial synthesis.

The polymeric microparticle can be prepared from a variety of polymerisable monomers, including styrenes, acrylates and unsaturated chlorides, esters, acetates, amides and alcohols, including, but not limited to, polystyrene (including high density polystyrene latexes such as brominated polystyrene), polymethylmethacrylate and other polyacrylic acids, polyacrylonitrile, polyacrylamide, polyacrolein, polydimethylsiloxane, polybutadiene, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidenechloride and polydivinylbenzene. The microparticles may be prepared from styrene monomers. Ceramic microparticles may be comprised of silica, alumina, titania or any other suitable transparent material. Preferably, silica particles are employed. A suitable method of making silica microparticles is described, for example in *"The Colloid Chemistry of Silica and Silicates"* (Cornell University Press) by Ralph K Iler 1955 and U.S. Patent No 5,439,624, the disclosures of which are incorporated herein by reference.

Fluorescent dyes may be incorporated into microparticles by any suitable method known in the art, such as copolymerisation of a polymerisable monomer and a dye-containing co-monomer or addition of a suitable dye derivative in a suitable organic solvent to an aqueous suspension as for example disclosed in Singer *et al.*, (*supra* including references cited therein), Campian *et al.* (1994, In *"Innovation and Perspectives on Solid Phase Synthesis"* Epton, R., Birmingham: Mayflower, 469-472, incorporated herein by reference) and Egner *et al.* (1997, *Chem. Commun.* 735-736, incorporated herein by reference). Alternatively, fluorescent microparticles may be produced having at least one fluorescent spherical zone. Such particles may be prepared as for example described in U.S. Patent No. 5,786,219 (Zhang *et al.*), which is incorporated herein by reference. In a preferred embodiment, one or more fluorescent dyes are incorporated within a microparticle. Compared to surface attachment of fluorescent dyes, incorporation of dyes within microparticles reduces the physical exposure of the fluorescent dye(s) to various

solvents used in combinatorial synthesis and thus increases the stability of the carrier-fluorescent dye complexes.

Microparticles may also be prepared comprising different polymeric materials and/ or different ceramic materials. For example, such microparticles may comprise a plurality of layers of one or more different polymers as for example described in Caruso *et al.* (1998, *J. Am. Chem. Soc.* **120**: 8523-8524), which is incorporated herein by reference. Polymeric particles of this type may be prepared having different refractive indices or opacities, which may be used as detectable attributes according to the present invention. Alternatively, microparticles may comprise a plurality of layers, preferably composite multilayers, of ceramic materials as for example described in van Blaaderen *et al.* (1992, *Langmuir* **8**: 2921-2931), which is incorporated herein by reference. The atomic ratio of different ceramic materials may be used as a detectable and/or quantifiable attribute of the invention. In this regard, reference may be made to U.S. Patent No 5,439,624, which discloses measurement of Si/Al ratio of particles, by wavelength dispersive spectroscopy.

Any suitable method of analysing fluorescence emission is encompassed by the present invention. In this regard, the invention contemplates techniques including, but not restricted to, 2-photon and 3-photon time resolved fluorescence spectroscopy as for example disclosed by Lakowicz *et al.* (1997, *Biophys. J.*, **72**: 567, incorporated herein by reference), fluorescence lifetime imaging as for example disclosed by Eriksson *et al.* (1993, *Biophys. J.*, **2**: 64, incorporated herein by reference), and fluorescence resonance energy transfer as for example disclosed by Youvan *et al.* (1997, *Biotechnology et alia* **3**: 1-18).

Luminescence and phosphorescence may result respectively from a suitable luminescent or phosphorescent label as is known in the art. Any optical means of identifying such label may be used in this regard.

Infrared radiation may result from a suitable infrared dye. Exemplary infrared dyes that may be employed in the invention include, but are not restricted to, those disclosed in Lewis *et al.* (1999, *Dyes Pigm.* **42**(2): 197), Tawa *et al.* (1998, *Mater. Res. Soc. Symp. Proc.* 488 (Electrical, Optical, and Magnetic Properties of Organic Solid-State Materials IV), 885-890), Daneshvar, *et al.* (1999, *J. Immunol. Methods* **226**(1-2): 119-128), Rapaport *et al.* (1999, *Appl. Phys. Lett.* **74**(3): 329-331) and Durig *et al.* (1993, *J. Raman*

Spectrosc. 24(5): 281-5), which are incorporated herein by reference. Any suitable infrared spectroscopic method may be employed to interrogate the infrared dye. For instance, fourier transform infrared spectroscopy as for example described by Rahman *et al.* (1998, *J. Org. Chem.*, 63: 6196, incorporated herein by reference) may be used in this regard.

Suitably, electromagnetic scattering may result from diffraction, reflection, polarisation or refraction of the incident electromagnetic radiation including light and X-rays. In this regard, the carriers may be formed of different materials to provide a set of carriers with varying scattering properties such as different refractive indexes as for example described *supra*. Any suitable art recognised method of detecting and/or quantifying electromagnetic scatter may be employed. In this regard, the invention also contemplates methods employing contrast variation in light scattering as, for example, described in van Helden and Vrij (1980, *Journal of Colloidal and Interface Science* 76: 419-433), which is incorporated herein by reference.

Of course it will be appreciated that attributes other than electromagnetic radiation-related attributes may be utilised. Such attributes include size and shape of the carrier. For example, carriers, preferably particles, more preferably microparticles, may be shaped in the form of spheres, cubes, rectangular prisms, pyramids, cones, ovoids, sheets or cylinders. Typically, when microparticles are employed, these preferably have a diameter of about 0.01 μm to about 150 μm. In this regard, electrical impedance across a carrier may be measured to provide an estimate of the carrier volume (*Coulter volume*).

Alternatively, a detectable and/or quantifiable attribute of the carrier may comprise one or more surface deformations of the carrier inclusive of pits, holes, hollows, grooves or notches or any combination thereof.

The attribute may also reside in a chromophoric label. Suitable carriers comprising such chromophores are described for example in Tentorio *et al.* (1980, *Journal of Colloidal and Interface Science* 77: 419-426), which is incorporated herein by reference. A suitable method for non-destructive analysis of organic pigments and dyes, using a Raman microprobe, microfluorometer or absorption microspectrophotometer, is decribed for example in Guineau, B. (1989, *Cent. Rech. Conserv. Documents Graph., CNRS, Paris, Fr. Stud. Conserv* 34(1): 38-44), which is incorporated herein by reference.

Alternatively, the attribute may comprise a magnetic material inclusive of iron and magnetite, or an attribute that is detectable by acoustic backscatter as is known in the art.

It will be understood from the foregoing that the number of carriers having different detectable codes will be dependent on the number of different detectable and/or quantifiable attributes integrally associated with the carriers. For example, code heterogeneity may be achieved simply by use of carriers of different shapes and/or sizes, and/or by use of carriers which are formed of different materials as described above. Alternatively, the code heterogeneity may be facilitated by use of carriers having different markers and/or different combinations of markers integrally associated therewith. Code heterogeneity may also be enhanced by use of carriers having two or more linked solid supports (*e.g.*, bead or particle).

The carriers of the invention are applicable to any type of chemical reaction that can be carried out on a solid support. Such chemical reaction includes, for example: -

1. [2 + 2] cycloadditions including trapping of butadiene;
2. [2 + 3] cycloadditions including synthesis of isoxazolines, furans and modified peptides;
3. acetal formation including immobilization of diols, aldehydes and ketones;
4. aldol condensation including derivatization of aldehydes, synthesis of propanediols;
5. benzoin condensation including derivatization of aldehydes;
6. cyclocondensations including benzodiazepines and hydantoins, thiazolidines, -turn mimetics, porphyrins, phthalocyanines;
7. Dieckmann cyclization including cyclization of diesters;
8. Diels-Alder reaction including derivitisation of acrylic acid ;
9. Electrophilic addition including addition of alcohols to alkenes;

This algorithm also makes use of *integer division* to conveniently determine which microsphere corresponds to which gridspace. Integer division is similar to normal division, except the answer is rounded down to the nearest integer, *e.g.*, 213/100 = 2 or 14/15 = 0.

Requirements

The real-time algorithm was developed for use with any number of parameters and is currently implemented for the five parameters available in this study. Any header information must be removed and data must also be saved as a tab-delimited text file in the following format:

<integer><\t><integer><\t><integer> ... etc ...<\n>

where each integer is the value for a particular parameter. Unlike the post-acquisition algorithm, no identification numbers are required for the first integer, as the data from each microsphere is not stored internally.

A further, as yet unfulfilled, requirement is to provide real-time control of the sorting mechanism of the flow cytometer via the computer running the real-time algorithm.

Description

Pre-encoding of microspheres: The real-time algorithm is applied during the analysis of the population of microspheres. User input is required to select the number of parameters and the width of the gridspaces on each parameter in order to create the array of integers. As described in Example 2 (*"Pre-encoding of optically diverse microspheres"*), all the gridspaces are initially empty and so their corresponding integer in the array is equal to zero.

As each microsphere is detected and analysed by the flow cytometer, the real-time algorithm determines which gridspace it corresponds to by using integer division of its parameter values to index the multidimensional array. If it is within the internal sorting region of the corresponding gridspace, and the gridspace is labelled empty (ie. zero), then a sort decision is made to collect that microsphere (hence the need for real-time control of the sorting mechanism). The label for that gridspace is then changed to full (ie. one). If it

is outside the internal sorting region or if the gridspace is labelled full already, then a sort decision is made to reject that microsphere. In this manner, only optically unique microspheres are collected from the total population of microspheres. Note that the time taken to make this sort decision must be less than the time for the microsphere to travel from the observation point to the droplet break-off point.

Recording synthetic history of microspheres: To keep track of these unique microspheres through the combinatorial synthesis, the real-time algorithm is applied when each batch is re-analysed by the flow cytometer. As the internal sorting region was only necessary for the creation of an optically unique population, the algorithm now only needs to determine which microspheres correspond to which gridspace for a given batch task. An array of character strings of length *n* (where *n* is the number of cycles of the split-and-mix process and each character represents a particular reagent) with a one-to-one correspondence with the array of gridspaces can then be updated to include the synthetic history of each microsphere. These tasks can be performed post-hoc however, and do not need to be performed during the analysis.

Results

Time measurements for the main loop in WriteArray™ were undertaken to determine if the processing time for each microsphere was constant, and thus independent of population size. It was predicted using orders of magnitude (Stubbs *et al.*, 1993, *supra*) that the relationship between processing time and the number of iterations of the main loop (equivalent to population size) is O(n). Figure 23 is the result of measurements using different numbers of iterations for different numbers of parameters. As predicted, algorithm processing time is proportional to the number of iterations, with a linear fit of $R^2 = 0.9981$ or better for each of the different numbers of parameters.

By plotting the slopes from Figure 23 against the number of parameters, a second plot was obtained in Figure 24 that gives an equation for the time for *one* iteration:

$$y = 0.419x + 0.0023 \qquad (5.2)$$

where y is the time in microseconds for one iteration and x is the number of parameters. Thus for seventeen parameters (maximum commercially available), the total time for one

iteration is a constant 7.125 μs. This number is favourably comparable to the sort decision time required in high-speed flow cytometer sorters (eg. 6.5 μs for Coulter Elite™).

The only theoretical limit of this algorithm is the maximum number of gridspaces capable of existing within the entire parameter space. This is demonstrated in Figure 25, in which the number of unique microspheres from a randomly generated population is plotted against population size.

The general form of this curve is given by:

$$y = A(1 - e^{-kt}) \qquad (5.3)$$

where A is equal to the total number of available gridspaces, which in this case is given by four parameters each subdivided into ten regions ($10^4 = 10000$).

Data from an optically diverse mixture of QFITC-coated 4 μm blue-greenF microspheres from Example 4 (see Figure 26) was pre-encoded in a two-parameter simulation to demonstrate the real-time algorithm. Using eight divisions along each parameter and user-defined values of rl = rh = 30 channel numbers, the parameter space was divided into gridspaces each of width 128x128 channel numbers (ie. 1024/8 = 128), with an internal sorting region of 68x68 channel numbers. As the QFITC-coated microspheres are only optically diverse for FL1 and FL3, the remaining parameters (FS, SS and FL2) were made identical for each microsphere in order to negate their influence. Hence, the maximum number of unique microspheres is 64. The real-time algorithm was modified to save the parameter data from optically unique microspheres into a text file that was later plotted and overlaid with a graphical representation of the gridspaces (Figure 27). Fifty-six of the available 64 internal sorting regions of the gridspaces were successfully occupied by a single microsphere extracted from the total population using the real-time algorithm.

<u>Conclusions – Real-time algorithm</u>

The real-time algorithm has the potential to handle the large number of microspheres required for large combinatorial libraries. It provides a constant sort decision time given by equation 5.2 that is independent of population size and dependent only on the number of parameters. Even for seventeen parameters the sort decision time is capable

of processing high sorting rates (7.125 μs microsphere^{-1}). In addition, the maximum number of unique microspheres is equal to the number of available gridspaces. As the volume occupied by the available gridspaces is equal to the entire parameter space, this is therefore the highest possible number.

In order to implement fully this algorithm though, an interface needs to be established between the flow cytometer electronics and a computer. Although these needs are highly specific, previous research by Dvorak *et al.* (1991, *J Microcomputer Appl*, **14**: 327-341) and Leary *et al.* (1993, *US Patent 5199576*; 1993, *US Patent 5204884*; 1997, *Cytometry*, **27**: 233-238; 1997, *Proc SPIE: Int Soc Opt Eng*, **2982**: 342-352) may prove to be useful. Dvorak *et al.* (1991, *supra*) describe an electronic interface between a flow cytometer and a microcomputer, and offer to supply complete working drawings for non-commercial use. This would be useful for determining sort decision times and developing supporting software in the future. Leary *et al.* (1993 – 1997, *supra*) have developed and patented a modified high-speed sorter that is dynamically "trained" to select the optimal sort characteristics such as drop delay and pulse integration time in order to obtain a given number of rare cells (eg. occurrence of 1 in 10^6 cells) within a given assurance level, eg. 95%. Some detailed workings of the modified high-speed sorter are also provided for possible assistance (Dvorak *et al.*, 1991, *supra*; Leary *et al.*, 1993, *supra*). Alternatively, both Kettman *et al.* (1998, *supra*) and Bigos *et al.* (1999, *supra*) have successfully modified the electronics of a flow cytometer to suit their specific needs.

Conclusions

In summary, the post-acquisition algorithm can be used without any modification to a flow cytometer to track optically unique microspheres through a combinatorial synthesis. Due to the $O(n^2)$ relationship between processing time and population size, and the self-limiting nature of the algorithm, it is recommended that it is unsuitable for the intended application of handling large combinatorial libraries.

General conclusions

The present method is predicated at least in part on the maximum library size that can be encoded and the sample throughput (*i.e.*, number of samples processed per day). The number of optically unique microspheres that are extracted as a function of population

size follows an asymptotic curve similar to Figure 25. As the number of microspheres detected per second is approximately constant, the population size can be expressed in units of time instead. A predictive equation for the number of optically unique microspheres, U, extracted from a population as a function of time has been developed and has the following general form:

$$U = N(1 - e^{-k\alpha\beta t}) \tag{5.4}$$

The variables in this equation can all be obtained from the equations and relationships defined throughout this study. Equation 5.4 is based on the general form of Equation 5.3, and N is thus equal to the total number of available gridspaces, given by a slight modification of Equation 2.6:

$$N = \frac{1024^{p}}{\prod_{i=1}^{p}(v_i)} \tag{5.5}$$

where p is equal to the number of available parameters and v_i is the width of each gridspace for the ith parameter. The width of each gridspace equals:

$$v_i = rl_i + rh_i + w_i \tag{5.6}$$

where rl_i and rh_i are the lower and higher ranges as defined in Example 2 (and experimentally determined in Example 4) and w_i is the width of the internal sort region in each gridspace for the ith parameter.

The value of w_i is also important for calculation of α, which represents the percentage of each gridspace occupied by the internal sort region. In Figure 25, rl and rh were equal to zero and hence $\alpha = 1$. This represents a maximum case where every microsphere that occupies a vacant gridspace is extracted. In more realistic scenarios where $\alpha < 1$, only microspheres that occupy the internal sort region of a vacant gridspace are extracted. Hence, α is a multiplying factor given by:

$$\alpha = \prod_{i=1}^{p}\left(\frac{w_i}{v_i}\right) \tag{5.7}$$

The value of β equals the number of microspheres processed per second, and therefore βt equals the population size. The maximum value of β is given by the inverse of Equation 5.2, *i.e.*, the number of microspheres of processed per second using the real-time algorithm.

The exponential coefficient, k, is directly proportional to the optical diversity, σ, of the population of microspheres and inversely proportional to N:

$$k \approx \sigma N^{1} \tag{5.8}$$

where $\sigma = 1$ for random data (highest possible optical diversity) and $\sigma = 0.28$ for the mixture of QFITC-coated and uncoated 4 μm blue-greenF microspheres (experimentally determined by varying N).

This cohesive series of equations allows the number of optically unique microspheres to be predicted for a given period of time. This is a valuable relationship as it allows the maximum library size (which is proportional to the number of unique microspheres) to be optimised by altering the variables involved in Equation 5.4. In addition, each of the variables, *e.g.*, rl, rh, β, p, can be determined or estimated using the concepts and experiments described in this study.

Therefore, the feasibility of the proposed strategy can be predicted using Equation 5.4. Using the number of fluorescence parameters from Bigos *et al.* (1999, *supra*), $p = 9$, while the values for rl and rh for these fluorophores can be estimated from Table E, thus $rl = 50$ and $rh = 10$. The value of β for nine parameters as given by Equation 5.2 is 265000 microspheres s^{-1}, however current maximum sorting rates in commercial flow cytometers are approximately 25000 microspheres s^{-1}, hence $\beta = 25000$ microspheres s^{-1}. The value of $k = 0.28N$ for the QFITC-coated microspheres is representative of the optical diversity attainable using the synthetic methods in Example 3. As α and N are dependent on the width of the internal sort region, w_i, Figure 28 displays the number of unique microspheres, U, as a function of w_I. The value of t is considered to be twenty-four hours for simplicity.

The maximum value of U for the above conditions therefore corresponds to an internal sort region width of 110. Note the sharp decrease from $w_I = 110$ to $w_i = 111$. The discontinuities present in Figure 28 are due to the integer divisions required in Equation

C:\'

32. Using $w_i = 110$, the number of unique microspheres is expressed as a function of time over a 24 hour period in Figure 29. Hence, after 24 hours, 7.02×10^6 unique microspheres could be extracted. The practicalities of maintaining a flow cytometer at 25000 microspheres s^{-1} for 24 hours would be difficult to overcome, *e.g.*, a constant supply of sheath fluid and sample would be required. However, 2.00×10^6 unique microspheres could be extracted in only 4 1/2 hours, a much more realistic time frame. The time for all further analyses is equal to U/β seconds as every microsphere is collected. Hence it is the pre-encoding step that is rate-determining for the proposed strategy.

Two million optically unique microspheres will allow for the combinatorial synthesis of all 65536 possible oligonucleotides of eight nucleotides in length. This library could then be used for DNA sequencing by hybridisation. The presence of multiple or redundant microspheres improves the overall robustness of the proposed strategy, as all the redundant microspheres with the same compound should return similar results in the final screening process. In order to increase the maximum library size, smaller values of *rl* and *rh*, as well as a higher degree of optical diversity would be necessary. This could be achieved by more effective fluorescence compensation and redispersion of the microspheres in the same solvent that is used as sheath fluid to avoid the initial downward shift for all parameters after the first sort.

EXAMPLE 6

Reproducibility between runs of seven aliquots of the same sample

A sample of 15 μm fluorescent silica microspheres in Milli-Q™ water (Micromod, Cat. No. 40-15401, 10 μg/mL, NH_2 functionalised) was prepared and passed through a FACSCalibur™ flow cytometer (Becton Dickinson). Scattering and fluorescence signals inside a region (Figure 30) for one million events were recorded. This was repeated with the same sample another six times, separated by 10 seconds between each run. The mean FL3 (red fluorescence), forward scatter and side scatter values were determined from all seven data sets. The average side scatter was 387 (standard deviation = 23, 6%), the average forward scatter was 3003 (standard deviation = 153, 5%) and the average FL3 value was 301 (standard deviation = 31, 10%). These results indicate that

there is a small variation in the mean values of the scattering/fluorescence signals from chosen sample.

EXAMPLE 7

Non fluorescent microspheres collected and repassed through the flow cytometer give reproducible scattering values

A sample of 10.2 µm polystyrene/divinylbenzene microspheres (Duke Scientific Corp., Cat. No. 7510A, CV = 14.7%, 10 µg/mL in water) was passed through the flow cytometer. Two regions were set up. Microspheres in Region 1 were detected as events (Figure 31, Panel A), but all were run to waste, except those in Region 2. The microspheres inside Region 2 were collected, reconcentrated by filtration in a size 5 filter (pore size 4 – 10 µm) and repassed through the flow cytometer (Figure 31, Panel B) after removing the Region 2 gate. The microspheres were then free to appear anywhere inside Region 1. As shown in Panel B of this figure, the microspheres reappeared in the place where Region 2 was removed.

EXAMPLE 8

Polystyrene 10.2 and 21 µm microsphere mixtures collected and repassed through the flow cytometer give reproducible scattering and fluorescence values

A mixture containing 10.2 µm polystyrene/divinylbenzene microspheres (Duke Scientific Corp., Cat. No. 7510A, CV = 14.7%, 10 µg/mL in water) and 21 µm polystyrene/divinylbenzene microspheres (Duke Scientific Corp., Cat. No. 7520A, CV = 14.7%, 10 µg/mL in water) was passed through the flow cytometer. Two regions were set up. Microspheres in Region 1 were detected as events (Figure 32, Panels A, B), but all were run to waste, except those in Region 2. The microspheres inside Region 2 were collected, reconcentrated by filtration in a size 5 filter (pore size 4 – 10 µm) and repassed through the flow cytometer (Figure 32, Panels C, D) after removing the Region 2 gate. The microspheres were then free to appear anywhere inside Region 1. As shown in Panel B of Figure 32, the microspheres reappeared in the place where Region 2 was removed.

These results show that microspheres can be collected and repassed through the flow cytometer reproducibly using light scattering as an attribute.

EXAMPLE 9

Fluorescent green polystyrene microspheres collected and repassed through the flow cytometer give reproducible scattering and fluorescence values

A sample of fluorescent green polystyrene microspheres (6 µm, Becton Dickinson Calibrite microspheres) was passed through the flow cytometer. Two regions were set up. Microspheres in Region 1 were detected as events (Figure 33, Panel A and B), but all were run to waste, except those in Region 2. The microspheres inside Region 2 were collected, reconcentrated by filtration in a size 5 filter (pore size 4 – 10 µm) and repassed through the flow cytometer (Figure 33, Panel C and D) after removing the Region 2 gate. The microspheres were then free to appear anywhere inside Region 1. As shown in Panel C, the microspheres reappeared in the place where Region 2 was removed. This shows that microspheres can be collected and repassed through the flow cytometer reproducibly using fluorescence as an attribute.

EXAMPLE 10

Non-fluorescent polystyrene/divinylbenzene (DVB) microspheres swelled in DMF for 3 hours and returned to Milli-Q water give scattering values similar to those that have not been subjected to DMF.

A sample of 10.2 µm polystyrene/divinylbenzene microspheres (Duke Scientific Corp., Cat. No. 7510A, CV = 14.7%, 10 µg/mL in Milli-Q™ water) was passed through the flow cytometer and the side scatter and forward scatter of 10000 events inside Region 1 were recorded (Figure 34, Panel A). The mean side scatter value was 1194 and the mean forward scatter value was 316.

A second sample of 10.2 µm polystyrene/divinylbenzene microspheres (Duke Scientific Corp., Cat. No. 7510A, CV = 14.7%) was stirred in pure DMF for three hours before being transferred gradually back into Milli-Q™ water. The sample (10 µg/mL in water) was passed through the flow cytometer and the side scatter and forward scatter of

10000 events inside Region 1 were recorded (Figure 34, Panel B). The mean side scatter value was 1224 and the mean forward scatter value was 316.

The mean forward scatter value for both samples was the same. The mean side scatter values were in close agreement, taking into account that a logarithmic scale was used and this normal kind of variation occurs in multiple runs of a sample.

EXAMPLE 11

Fluorescent red silica microspheres swelled in DMF for 3 hours and returned to Milli-Q water give scattering and fluorescence values similar to those that have not been subjected to DMF.

A sample of 15 µm fluorescent silica microspheres (Micromod, Cat. No. 40-15401, 10 µg/mL in water, NH_2 functionalised) was passed through the flow cytometer and the side scatter and FL3 (red fluorescence) of 10000 events inside Region 1 were recorded (Figure 35, Panel A). Rhodamine B dye had been incorporated into the silica microspheres during microsphere synthesis. The mean FL3 (red fluorescence) value was 376 and the mean side scatter value was 253.

A second sample of 15 µm fluorescent silica microspheres (Micromod, Cat. No. 40-15401, 10 µg/mL in water, NH_2 functionalised) was stirred in pure DMF for three hours before being transferred gradually back into Milli-Q™ water. The sample (10 µg/mL in water) was passed through the flow cytometer and the side scatter and forward scatter of 10000 events inside Region 1 were recorded (Figure 35, Panel B). The mean FL3 (red fluorescence) value was 379 and the mean side scatter value was 218.

EXAMPLE 12

Polystyrene/DVB microspheres that have undergone amino acid couplings give similar scattering and fluorescence values to those that have not been subjected to coupling.

A sample of 20 µm Tentagel microspheres (Rapp Polymere GmbH, Tentagel M-NH_2, Cat. no. M 30 202, 10 mg) in Milli-Q™ water was prepared. This was carried out by first sonicating the microspheres in DCM for 10 minutes, transferring them gradually to

DMF, followed by gradual transfer to Milli-Q™ water. This method prevented clumping of the microspheres. The sample was passed through the flow cytometer and those in Region 1 were collected (Figure 36, Panel A).

A second sample of 20 µm Tentagel microspheres (Rapp Polymere GmbH, Tentagel M-NH_2, Cat. no. M 30 202, 10 mg) was subjected to an amino acid coupling and then run through the flow cytometer. To prepare the sample, the microspheres were sonicated in DCM for 10 minutes and transferred gradually to DMF. Amino acid coupling to microspheres was performed using normal Fmoc chemistry (10 minutes with 150 mg Fmoc-Glycine-OH (Novabiochem), 1 mL HBTU and 120 µL DIEA). The microspheres were washed with DMF and gradually transferred to Milli-Q water. The microspheres reappeared inside region 1 when passed through the flow cytometer using the same instrument settings (Figure 36, Panel B).

A third sample of 20 µm Tentagel microspheres (Rapp Polymere GmbH, Tentagel M-NH_2, Cat. no. M 30 202, 10 mg) was subjected to three amino acid couplings and then run through the flow cytometer. To prepare the sample, the microspheres were sonicated in DCM for 10 minutes and transferred gradually to DMF. Amino acid coupling to microspheres was performed using normal Fmoc chemistry (10 minutes with 150 mg Fmoc-Glycine-OH (Novabiochem), 1 mL HBTU and 120 µL DIEA). The microspheres were washed with DMF and the Fmoc protecting group was removed from the microspheres using piperidine/DMF (1:1) for 6 minutes. After washing with DMF, a second amino acid coupling was performed using normal Fmoc chemistry (10 minutes with 160 mg Fmoc-Alanine-OH (Novabiochem), 1 mL HBTU and 120 µL DIEA), followed by more DMF washing. The deprotection using piperidine/DMF was repeated and a third coupling was performed (10 minutes with 150 mg Fmoc-Glycine-OH (Novabiochem), 1 mL HBTU and 120 µL DIEA). After washing with DMF, the microspheres were transferred gradually to Milli-Q™ water and run through the flow cytometer. The microspheres reappeared inside region 1 when passed through the flow cytometer using the same instrument settings (Figure 36, Panel C).

EXAMPLE 13

Fluorescent red silica microspheres collected, reacted with an amino acid and repassed through the flow cytometer give side scatter and red fluorescence values before and after coupling.

A sample of 15 µm fluorescent silica microspheres in Milli-Q™ water (Micromod, Cat. No. 40-15401, 10 µg/mL, NH_2 functionalised) was prepared and passed through the flow cytometer. Scattering and fluorescence signals inside Region 1 (Figure 37, Panels A and C; microcapsglyrerun and microcapsglyrerunfs) for one million events were recorded and these particles were collected in a 50-mL centrifuge tube. The microspheres were concentrated by filtering through a size 5 filter (pore size 4 – 10 µm) and gradually transferred to DMF. Amino acid coupling to microspheres was performed using normal Fmoc chemistry (10 minutes with 150 mg Fmoc-Glycine-OH (Novabiochem), 1 mL HBTU and 120 µL DIEA). The microspheres were washed with DMF and gradually transferred to Milli-Q™ water. The microspheres reappeared inside region 1 when passed through the flow cytometer using the same instrument settings (Panels B and D).

EXAMPLE 14

Method of increasing optodiversity by permanently attaching small fluorescent microspheres to carriers.

Polystyrene/divinylbenzene microspheres (10.2 µm Duke Scientific Corp., Cat. No. 7510A, CV = 14.7%, 10 µg/mL in Milli-Q™ water) are mixed with green fluorescent microspheres (0.2 µm, Molecular Probes, latex, 20 µL). The red microspheres adhere to the larger microspheres and the excess small microspheres are removed by washing with Milli-Q™ water (5 x 20 mL).

To increase adherence, the fluorescent microspheres are coated with multilayers of polyelectrolyte prior to mixing with the 10.2 µm carriers. The procedure for coating the microspheres involves soaking for 24 hours in a 1% solution of polyethyleneimine (a positively charged polyelectrolyte), washing with Milli-Q™ water, soaking for 24 hours in a 1% solution of polyacrylic acid (a negatively charged polyelectrolyte), and washing.

The carriers with the small fluorescent particles attached are passed through the flow cytometer and FL1 (green fluorescence) and forward scatter are measured (Figure 38, Panel A). If orange or red fluorescent microspheres are used instead of green, the FL1 values of the carriers change (Figure 38, Panel B and C).

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Those of skill in the art will therefore appreciate that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention. All such modifications and changes are intended to be included within the scope of the appendant claims.

CLAIMS

1. A carrier on which a compound can be synthesised, wherein said carrier has at least two attributes integrally associated therewith, which attributes are detectable and/or quantifiable during synthesis of the compound and which define a code identifying the carrier before, during and after said synthesis, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier.

2. The carrier of claim 1, wherein at least one of said attributes is comprised within or internally of the carrier.

3. The carrier of claim 1, wherein at least one of said attributes is an electromagnetic radiation-related attribute.

4. The carrier of claim 3, wherein the electromagnetic radiation-related attribute is selected from the group consisting of fluorescence emission, luminescence, phosphorescence, infrared radiation, electromagnetic scattering including light and X-ray scattering, light transmittance, light absorbance and electrical impedance.

5. The carrier of claim 3, wherein the electromagnetic radiation-related attribute is a light emitting, light transmitting or light absorbing attribute detectable by illuminating the carrier with incident light of one or more selected wavelengths or of one or more selected vectors.

6. The carrier of claim 1, having at least three detectable and/or quantifiable attributes integrally associated therewith.

7. The carrier of claim 3, wherein the electromagnetic radiation-related attribute comprises a fluorescent dye.

8. The carrier of claim 1, wherein the carrier is a colloidal particle.

9. The carrier of claim 1, wherein the carrier is a colloidal particle in the form of a pellet, disc, capillary, hollow fibre needle, pin or chip.

10. The carrier of claim 9, wherein the colloidal particle is a polymeric or ceramic particle.

11. The carrier of claim 10, wherein the ceramic particle is a silica particle.

12. The carrier of claim 10, wherein the ceramic particle has a diameter of about 0.01 μm to about 150 μm.

13. The carrier of claim 1, having a shape selected from the group consisting of spheres, cubes, rectangular prisms, pyramids, cones, ovoids, sheets or cylinders.

14. The carrier of claim 1, wherein the carrier comprises functionalities selected from the group consisting of $-NH_2$, -COOH, -SOH, -SSH and sulfate.

15. A plurality of carriers on which a plurality of different compounds can be synthesised, including a population of detectably distinct carriers each having a code, which distinctively identifies a respective carrier before, during and after said synthesis from other carriers, and which is characterised by at least two detectable and/or quantifiable attributes integrally associated with the carrier, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier.

16. The plurality of carriers of claim 15, wherein at least one of said attributes of a respective carrier is comprised within or internally of the carrier.

17. The plurality of carriers of claim 15, wherein at least one of said attributes of a respective carrier is an electromagnetic radiation-related attribute.

18. The plurality of carriers of claim 17, wherein the electromagnetic radiation-related attribute is selected from the group consisting of fluorescence emission, luminescence, phosphorescence, infrared radiation, electromagnetic scattering including light and X-ray scattering, light transmittance, light absorbance and electrical impedance.

19. The plurality of carriers of claim 17, wherein the electromagnetic radiation-related attribute is a light emitting, light transmitting or light absorbing attribute detectable by illuminating the carrier with incident light of one or more selected wavelengths or of one or more selected vectors.

20. The plurality of carriers of claim 15, wherein a respective carrier has at least three detectable and/or quantifiable attributes integrally associated therewith.

21. The plurality of carriers of claim 17, wherein the electromagnetic radiation-related attribute of a respective carrier comprises a fluorescent dye.

22. The plurality of carriers of claim 15, wherein each carrier is a colloidal particle.

23. The plurality of carriers of claim 15, wherein the carriers have different shapes selected from the group consisting of spheres, cubes, rectangular prisms, pyramids, cones, ovoids, sheets or cylinders.

24. The plurality of carriers of claim 15, wherein the carriers have different forms selected from the group consisting of pellet, disc, capillary, hollow fibre needle, pin and chip.

25. The plurality of carriers of claim 15, wherein the carriers have different sizes.

26. The plurality of carriers of claim 22, wherein the colloidal particle is a polymeric or ceramic particle.

27. The plurality of carriers of claim 26, wherein the ceramic particle is a silica particle.

28. The plurality of carriers of claim 26, wherein the carriers comprise ceramic particles with different diameters selected from about 0.01 μm to about 150 μm.

29. The plurality of carriers of claim 15, wherein a respective carrier comprises functionalities selected from the group consisting of $-NH_2$, -COOH, -SOH, -SSH and sulfate.

30. A method of producing a plurality of carriers including a population of detectably distinct carriers, comprising the steps of: -

(a) preparing a plurality of carriers having different codes wherein each code is characterised by at least two detectable and/or quantifiable attributes integrally associated with a respective carrier;

(b) detecting and/or quantifying the said attributes of each carrier to thereby assign a code for each carrier;

(c) identifying carriers having distinctive codes;

(d) identifying carriers having similar codes; and

(e) sorting the carriers having distinctive codes from the carriers having non-distinctive codes to thereby provide a plurality of carriers including a population having detectably distinct codes.

31. The method of claim 30, wherein step (a) is characterised in that the carriers are prepared by a split-process-recombine procedure.

32. The method of claim 30, wherein step (a) is characterised in that said least two attributes of a respective carrier results from a split-process recombine procedure.

33. The method of claim 32, wherein step (a) is further characterised in that one or more of the said at least two attributes of a respective carrier is layered onto the carrier.

34. The method of claim 30, wherein step (b) is characterised in that at least three different detectable and/or quantifiable attributes of a respective carrier are detected and/or quantified for code recordal.

35. The method of claim 30, wherein at least one of said attributes of a respective carrier is comprised within, or internally of, the carrier.

36. The method of claim 30, wherein at least one of said attributes of a respective carrier is an electromagnetic radiation-related attribute.

37. The method of claim 36, wherein step (b) is further characterised in that the electromagnetic radiation-related attribute is selected from the group consisting of fluorescence emission, luminescence, phosphorescence, infrared radiation, electromagnetic scattering including light and X-ray scattering, light transmittance, light absorbance and electrical impedance.

38. The method of claim 36, wherein step (b) is further characterised in that the electromagnetic radiation-related attribute is interrogated by illuminating the carrier with incident light of one or more selected wavelengths or of one or more selected vectors.

39. A method of synthesising and deconvoluting a combinatorial library comprising the steps of: -

(a) apportioning in a stochastic manner among a plurality of reaction vessels a plurality of carriers on which a plurality of different compounds can be synthesised, wherein said plurality of carriers includes a population of detectably distinct carriers each having a code, which distinctively identifies a respective carrier before, during and after said synthesis from other carriers, and which is characterised by at least two detectable and/or quantifiable attributes integrally associated with the carrier, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier;

(b) determining and recording the codes of said plurality of carriers in order to track the movement of individual detectably distinct carriers into particular reaction vessels of said plurality of reaction vessels, wherein said codes are determined prior to step (d);

(c) reacting the carriers in each reaction vessel with a synthon;

(d) pooling the carriers from each reaction vessel;

(e) apportioning the carriers in a stochastic manner among the plurality of reaction vessels;

(f) reacting the carriers in each reaction vessel with another synthon;

(g) recording the codes of said plurality of carriers in order to track the movement of individual detectably distinct carriers into particular reaction vessels of said plurality of reaction vessels, wherein said codes are recorded after step (e) or step (f);

(h) pooling the carriers from each reaction vessel; and

(i) iterating steps (e) through (h) as necessary to create a combinatorial compound library wherein member compounds of the library are associated with the detectably distinct carriers and wherein codes of the detectably distinct carriers are deconvolutable using tracking data provided by said recordal steps to identify the sequence of reactions experienced by the said detectably distinct carriers.

40. The method of claim 39, wherein the codes of the plurality of carriers are determined prior to step (d).

41. The method of claim 39, wherein steps (b) and (g) are further characterised in that at least three different detectable and/or quantifiable attributes of a respective carrier are detected and/or quantified for code recordal.

42. The method of claim 39, wherein at least one of said attributes of a respective carrier is comprised within, or internally of, the carrier.

43. The method of claim 39, wherein at least one of said attributes of a respective carrier is an electromagnetic radiation-related attribute.

44. The method of claim 43, wherein steps (b) and (g) are further characterised in that the electromagnetic radiation-related attribute is selected from the group consisting of fluorescence emission, luminescence, phosphorescence, infrared radiation, electromagnetic scattering including light and X-ray scattering, light transmittance, light absorbance and electrical impedance.

45. The method of claim 43, wherein steps (b) and (g) are further characterised in that the electromagnetic radiation-related attribute is interrogated by illuminating the carrier with incident light of one or more selected wavelengths or of one or more selected vectors.

46. The method of claim 39, wherein at least steps (g) and (e) are performed in a flow cytometer.

47. A combinatorial compound library produced by the method of any one of claims 39 to 46.

48. A kit comprising: -

(a) a combinatorial compound library including a plurality of different compounds wherein each compound is attached to at least one of a plurality of carriers, which includes a population of detectably distinct carriers each having a distinctive code, which distinctively identifies a respective carrier before, during and after synthesis of a corresponding compound from other carriers, and which is characterised by at least two detectable and/or quantifiable attributes integrally associated with the carrier, with the proviso that one of said attributes is other than shape, or surface deformation(s) of the carrier; and

(b) tracking data on each distinctive code to identify the sequence of reactions experienced by a respective detectably distinct carrier.

49. The kit of claim 48, wherein at least one of said attributes of a respective carrier is comprised within or internally of the carrier.

50. The kit of claim 48, wherein at least one of said attributes of a respective carrier is an electromagnetic radiation-related attribute.

51. The kit of claim 50, wherein the electromagnetic radiation-related attribute is selected from the group consisting of fluorescence emission, luminescence, phosphorescence, infrared radiation, electromagnetic scattering including light and X-ray scattering, light transmittance, light absorbance and electrical impedance.

52. The kit of claim 50, wherein the electromagnetic radiation-related attribute is a light emitting, light transmitting or light absorbing attribute detectable by illuminating the carrier with incident light of one or more selected wavelengths or of one or more selected vectors.

53. The kit of claim 48, wherein a respective carrier has at least three detectable and/or quantifiable attributes integrally associated therewith.

54. The kit of claim 48, wherein the electromagnetic radiation-related attribute of a respective carrier comprises a fluorescent dye.

55. The kit of claim 48, wherein each carrier is a colloidal particle.

56. The kit of claim 48, wherein the carriers have different shapes selected from the group consisting of spheres, cubes, rectangular prisms, pyramids, cones, ovoids, sheets or cylinders.

57. The kit of claim 48, wherein the carriers have different forms selected from the group consisting of pellet, disc, capillary, hollow fibre needle, pin and chip.

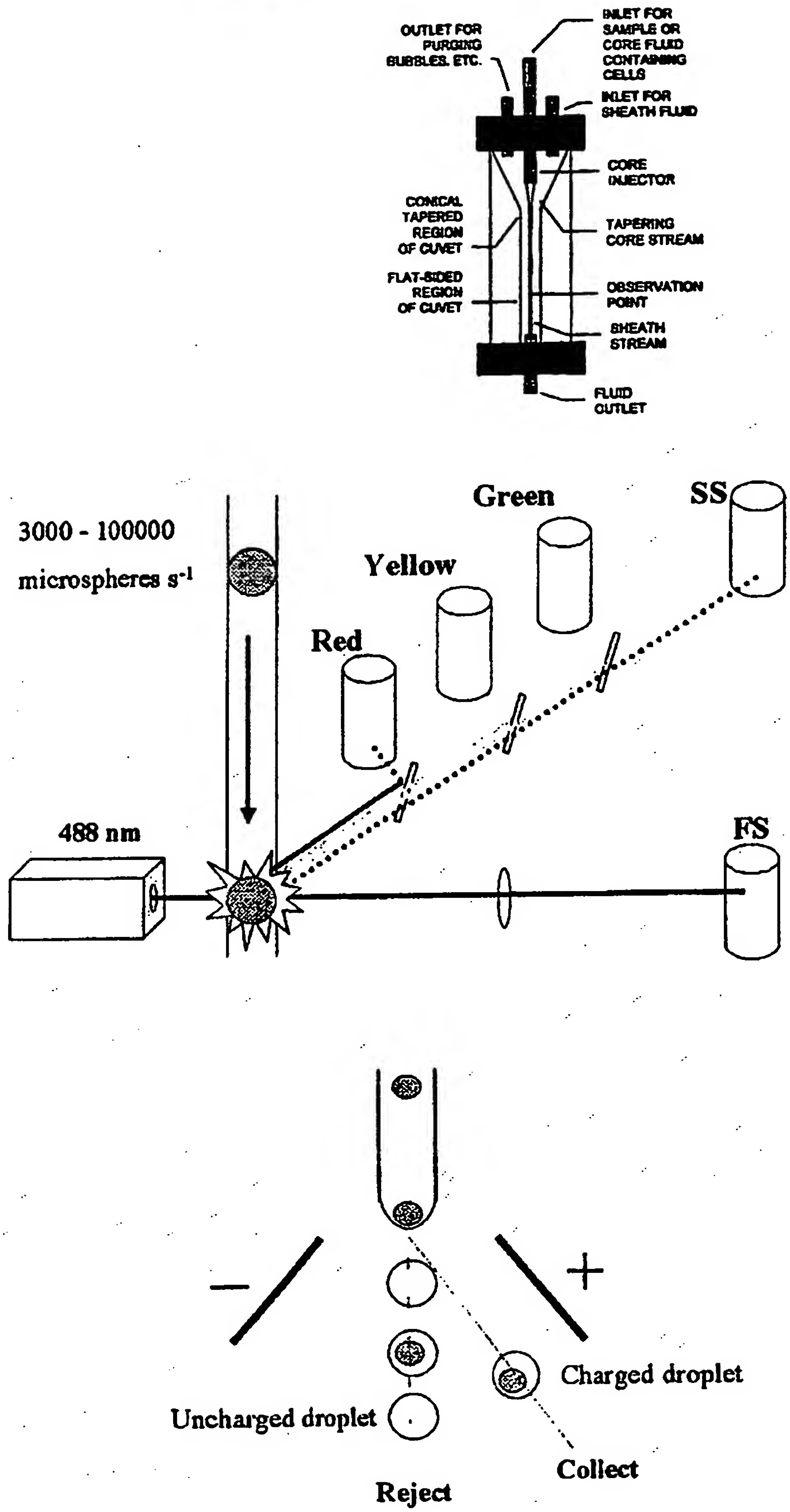
58. The kit of claim 48, wherein the carriers have different sizes.

59. The kit of claim 55, wherein the colloidal particle is a polymeric or ceramic particle.

60. The kit of claim 59, wherein the ceramic particle is a silica particle.

61. The kit of claim 59, wherein the carriers comprise ceramic particles with different diameters selected from about 0.01 μm to about 150 μm.

62. The kit of claim 48, wherein a respective carrier comprises functionalities selected from the group consisting of $-NH_2$, -COOH, -SOH, -SSH and sulfate.


OUTLET FOR PURGING BUBBLES, ETC.
INLET FOR SAMPLE OR CORE FLUID CONTAINING CELLS
INLET FOR SHEATH FLUID
CORE INJECTOR
CONICAL TAPERED REGION OF CUVET
TAPERING CORE STREAM
FLAT-SIDED REGION OF CUVET
OBSERVATION POINT
SHEATH STREAM
FLUID OUTLET
3000 - 100000 microspheres s-1
Green
SS
Yellow
Red
488 nm
FS
−
+
Charged droplet
Uncharged droplet
Collect
Reject

A

B

C

Figure 1

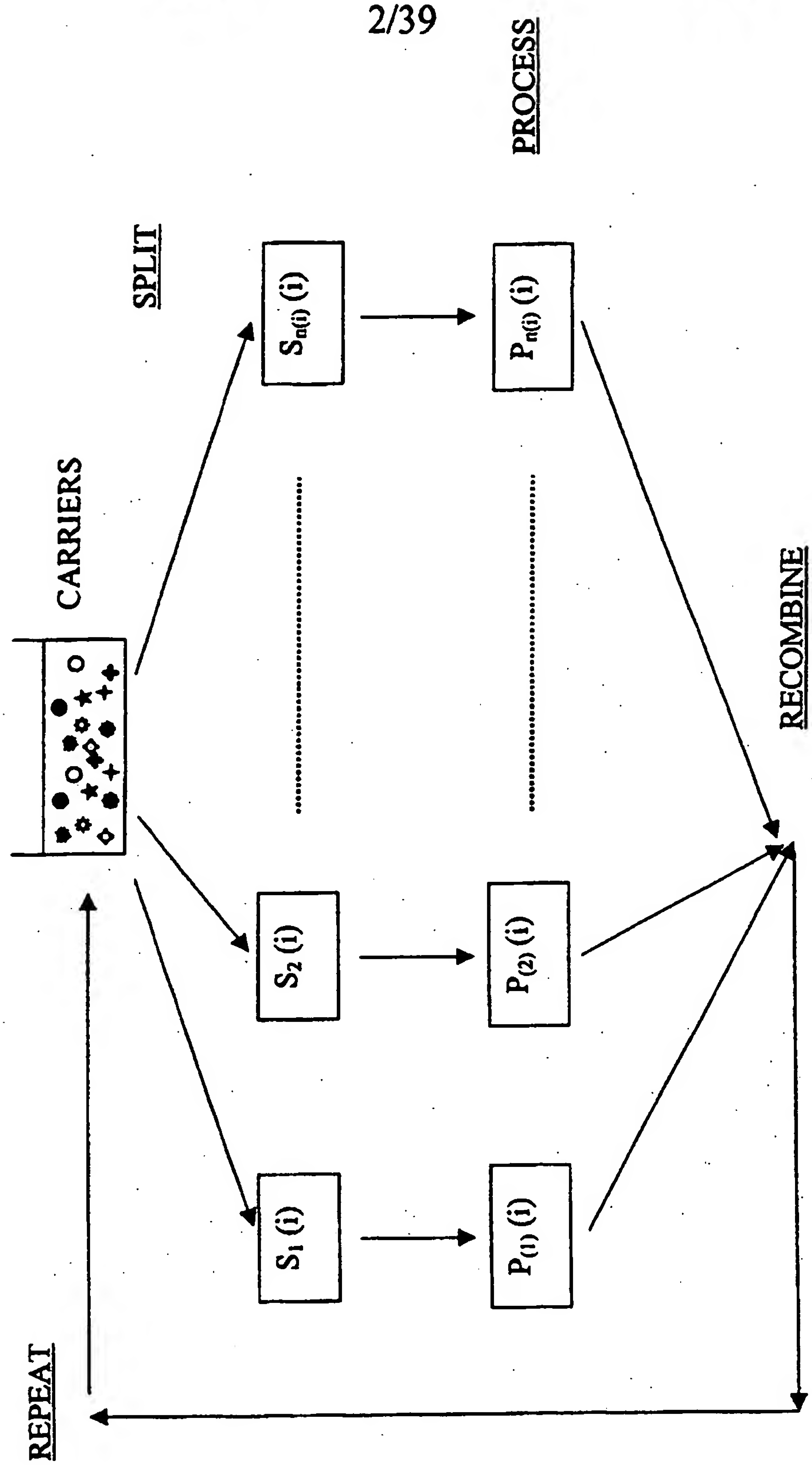

PROCESS
SPLIT
CARRIERS
$S_{n(i)}$ (i)
$P_{n(i)}$ (i)
RECOMBINE
S_2 (i)
$P_{(2)}$ (i)
S_1 (i)
$P_{(1)}$ (i)
REPEAT

Figure 2

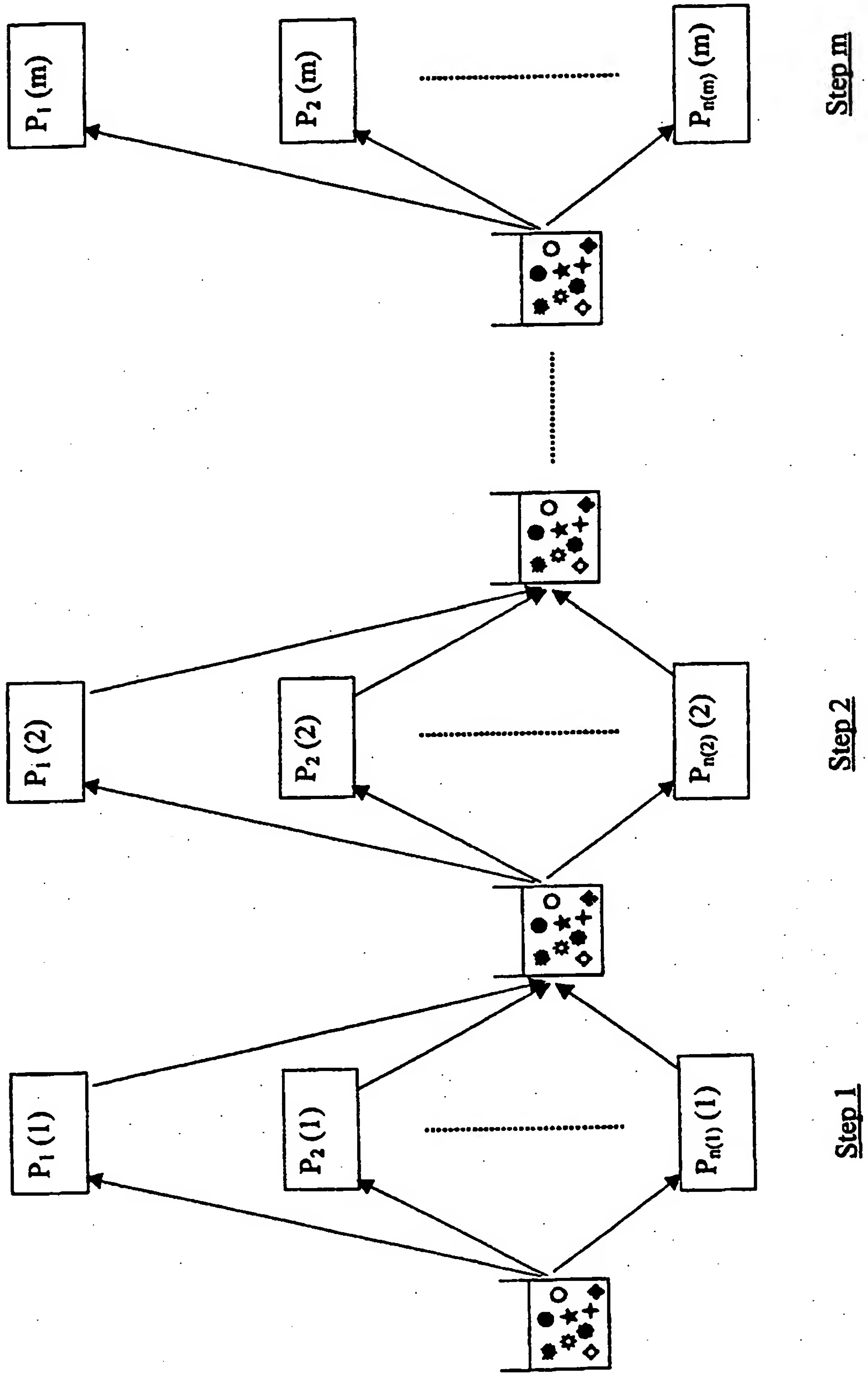


Figure 3

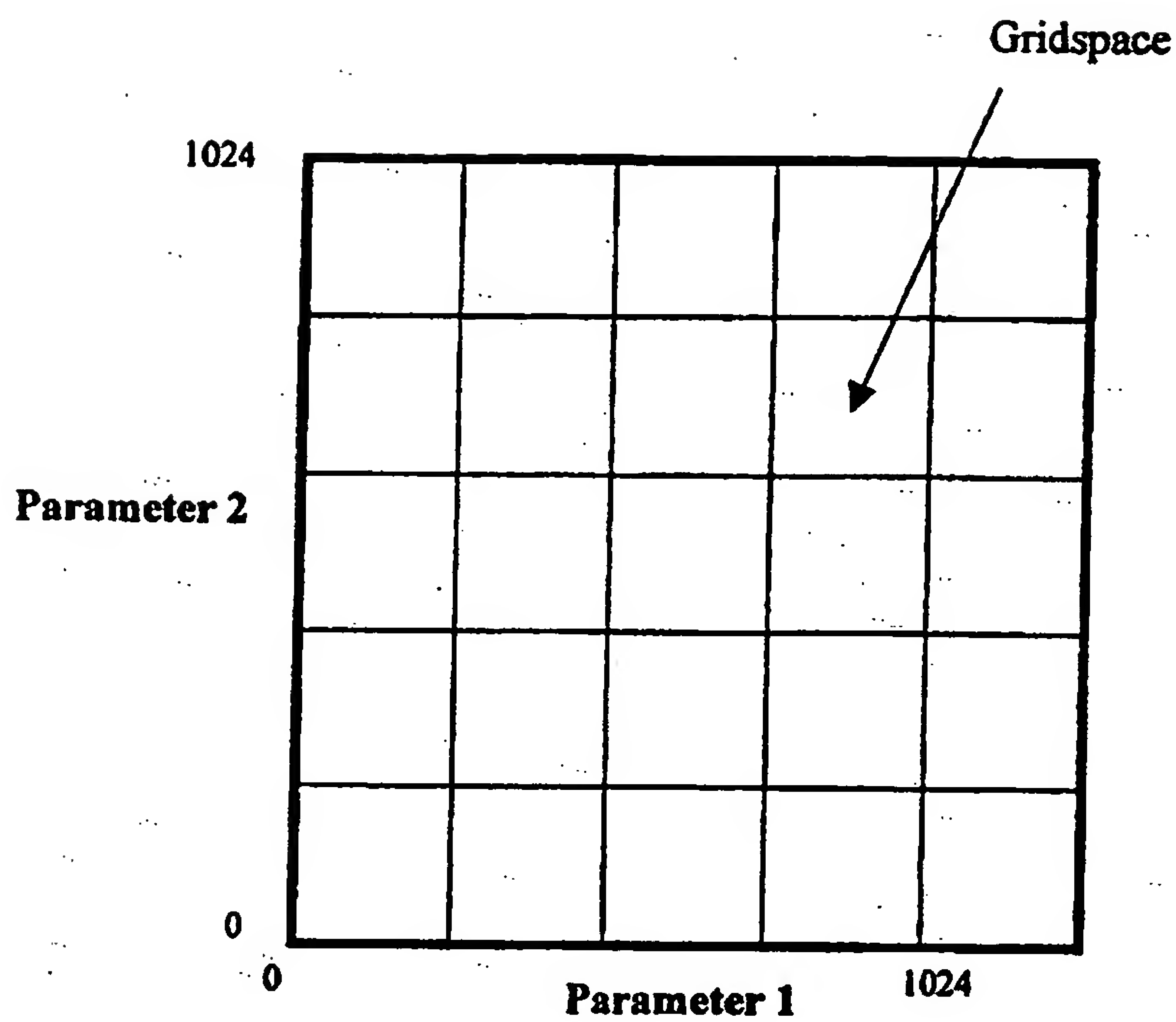


Figure 4

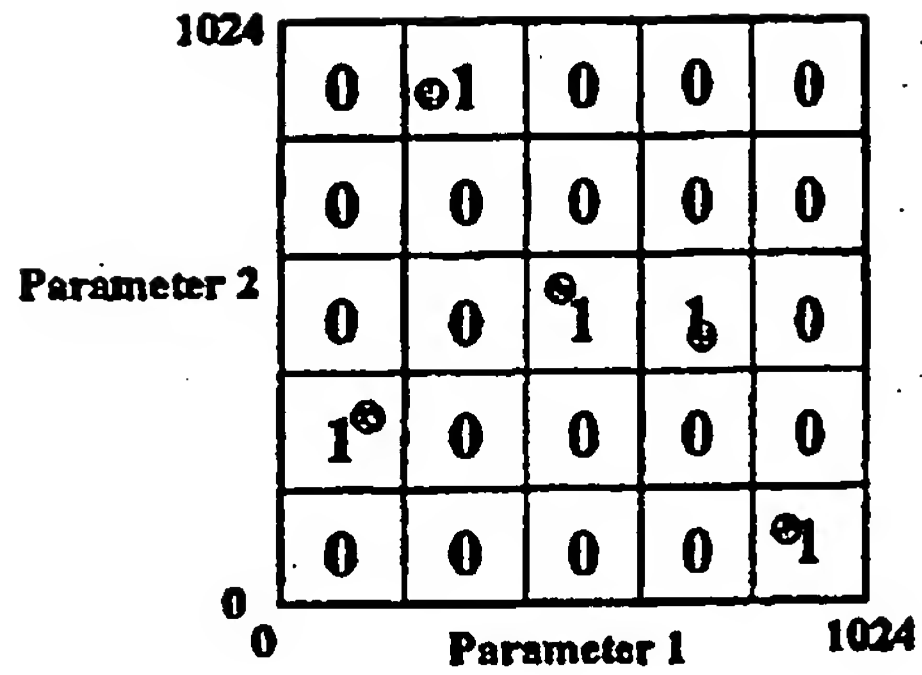


Figure 5.2(a)

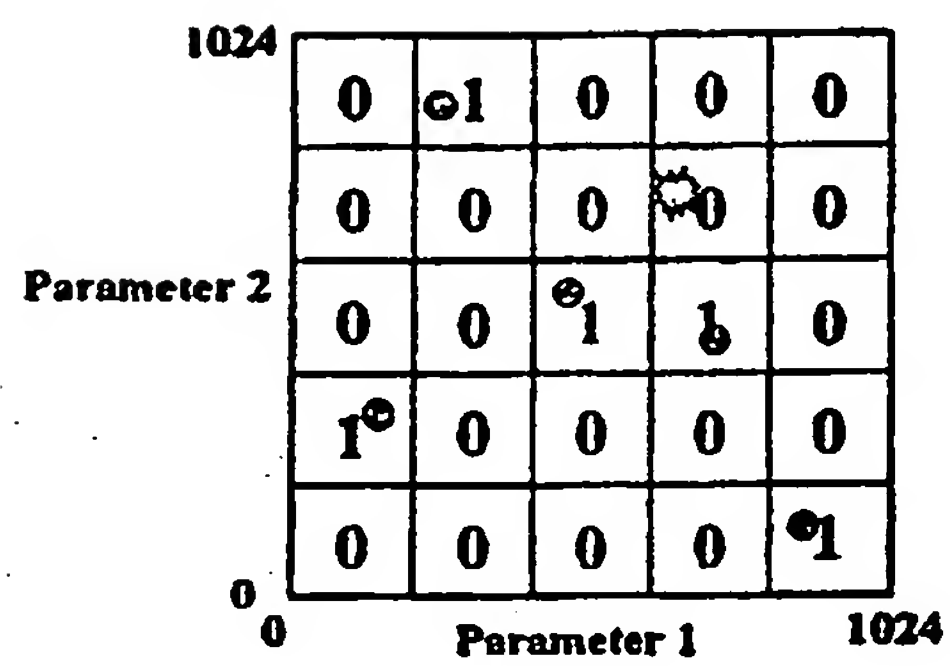


Figure 5.2(b)

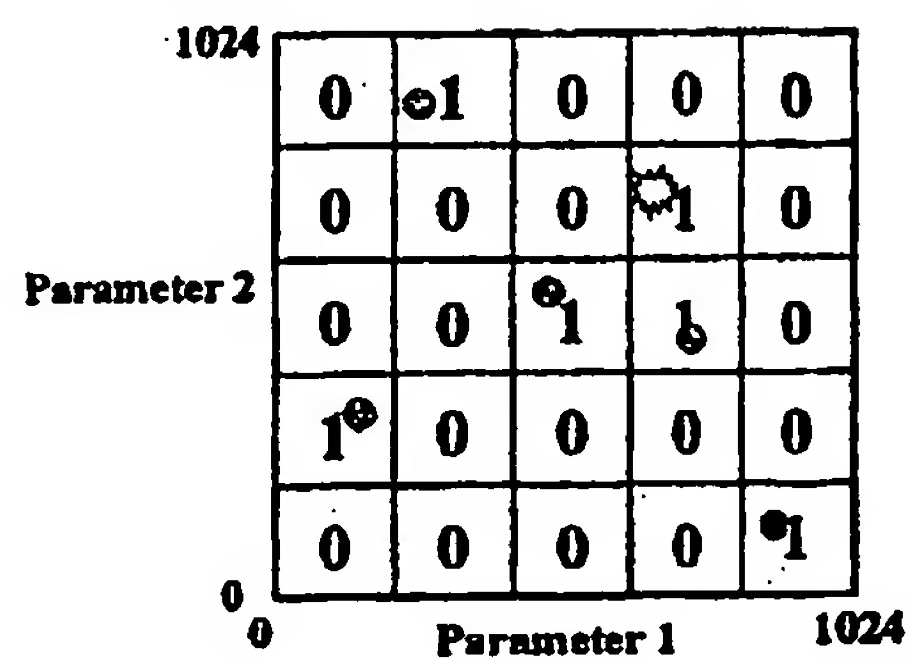


Figure 5.2(c)

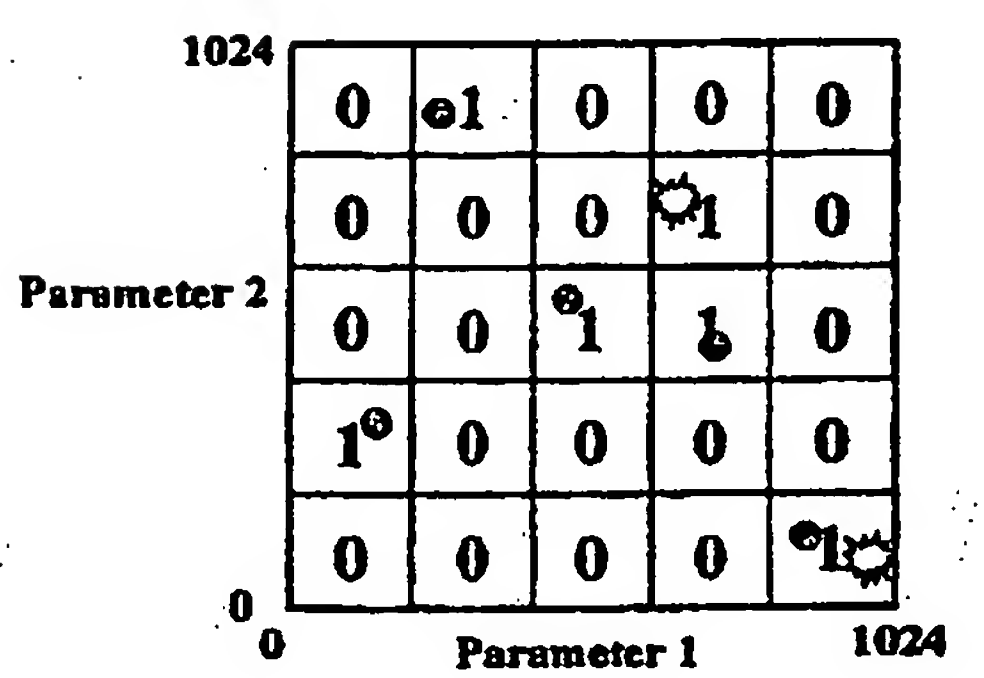


Figure 5.2(d)

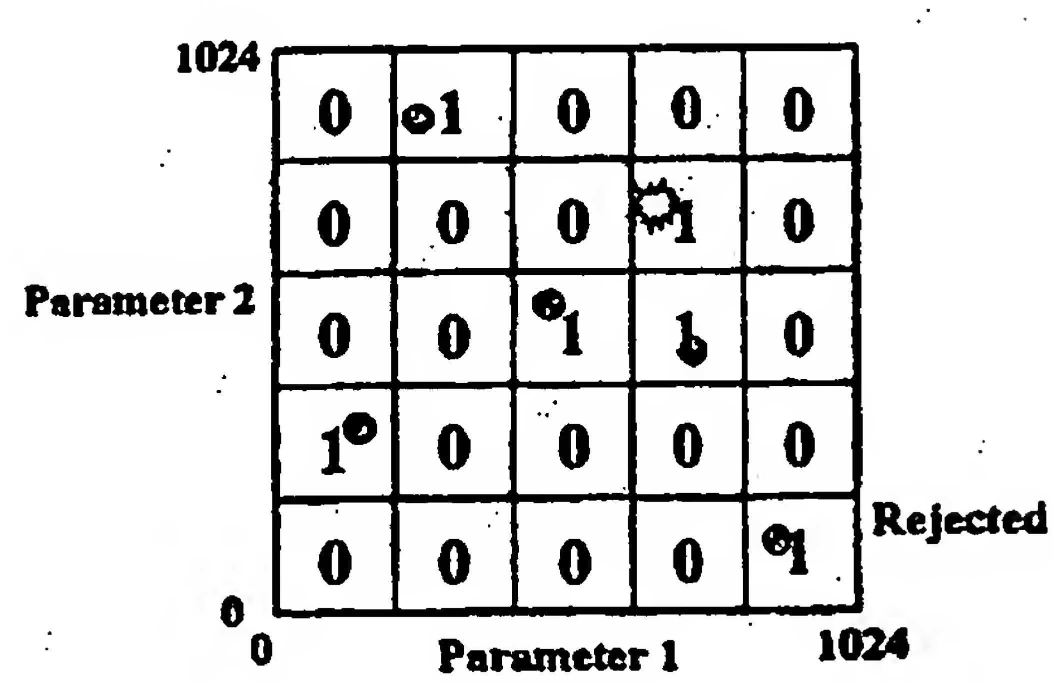


Figure 5.2(e)

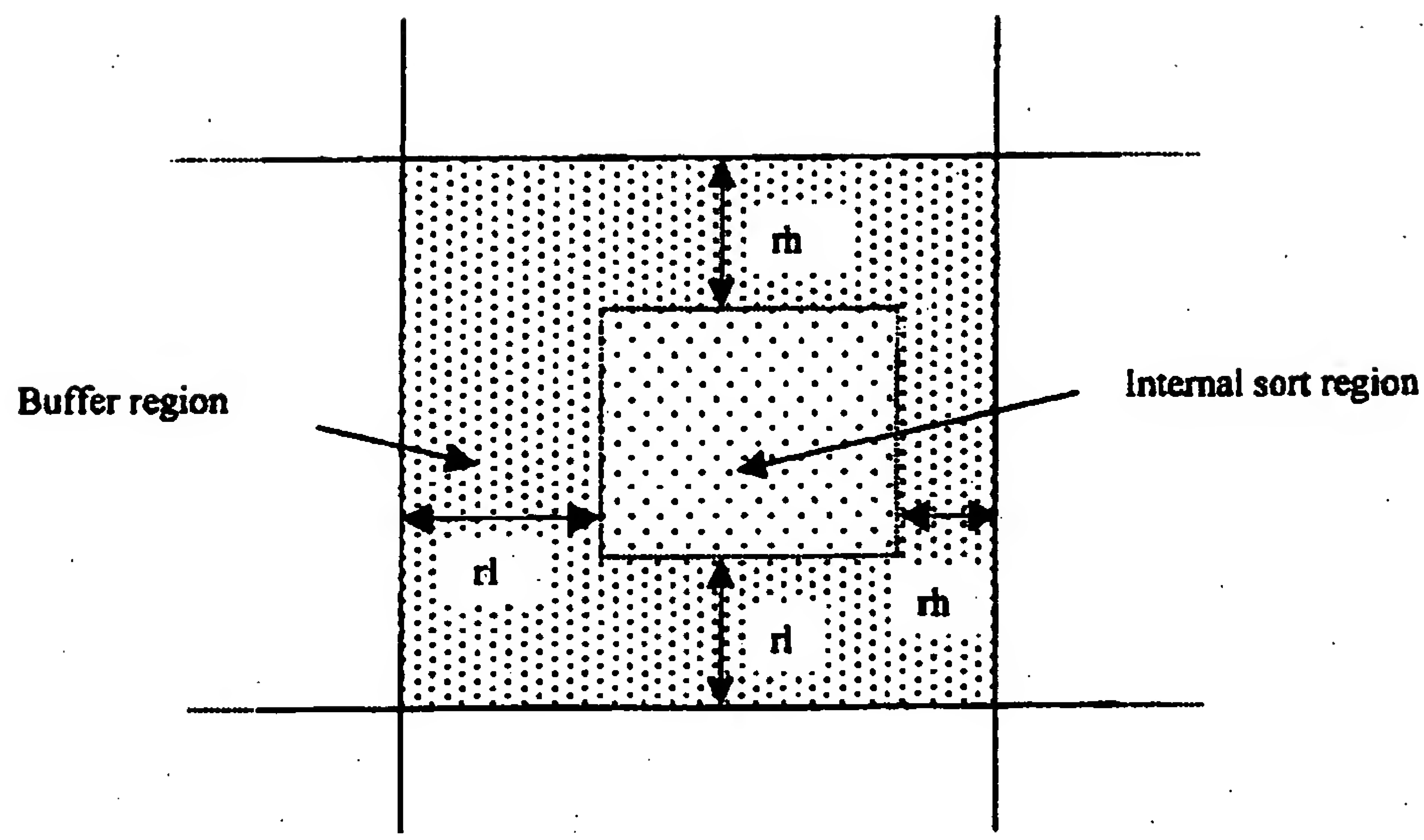
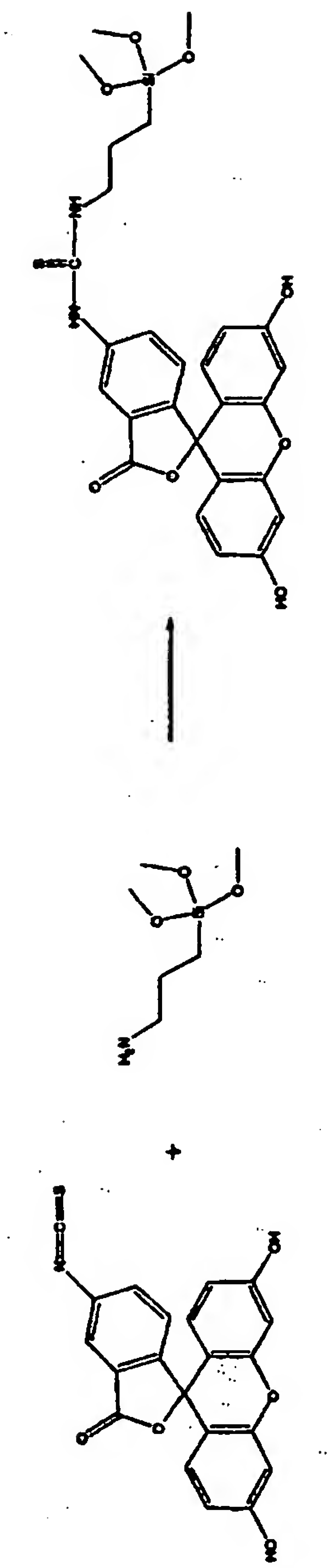


Figure 6



Fluorescein isothiocyanate

3-aminopropyltrimethoxysilane

Thiourea

Figure 7

Figure 8(a)

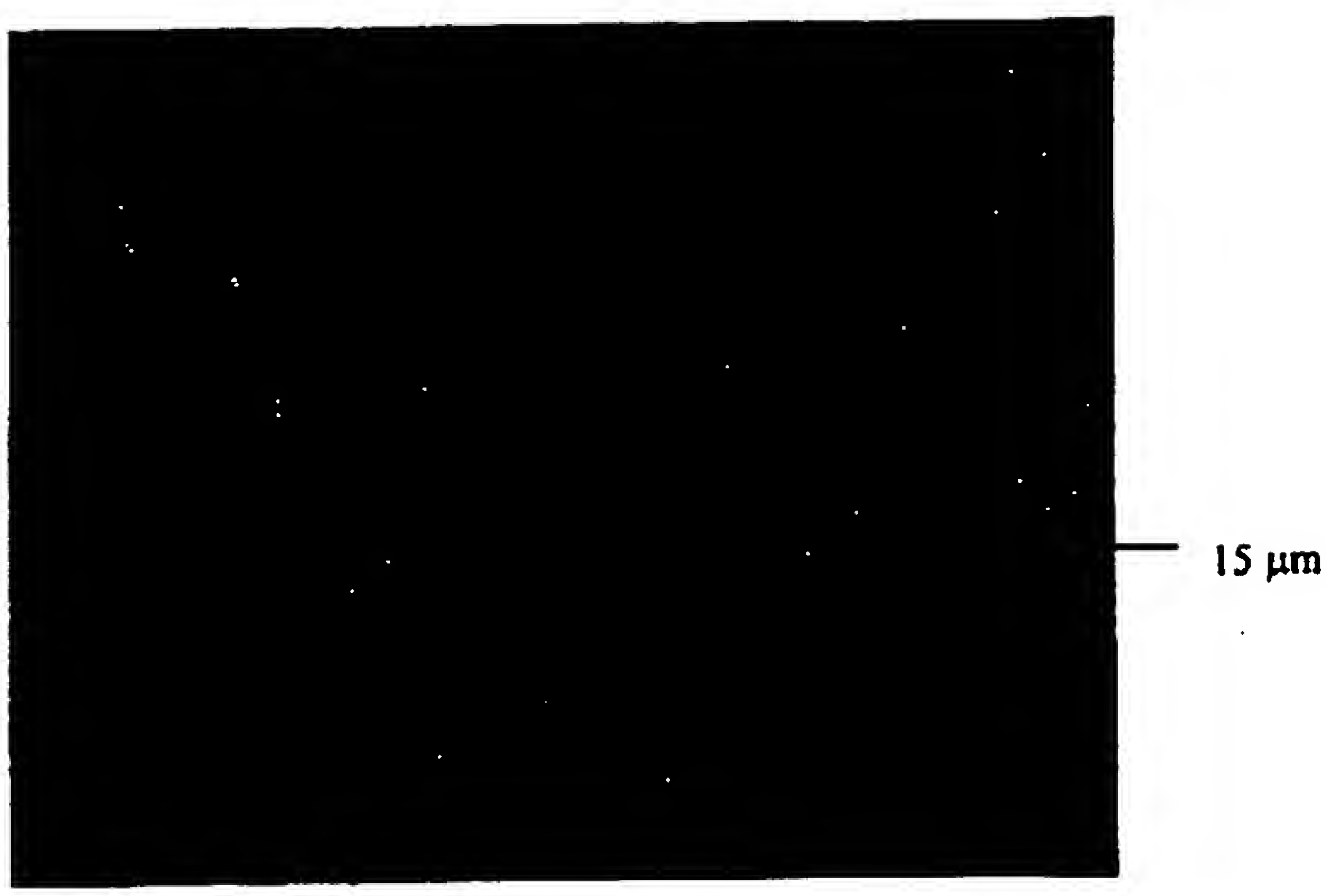


Figure 8(b)

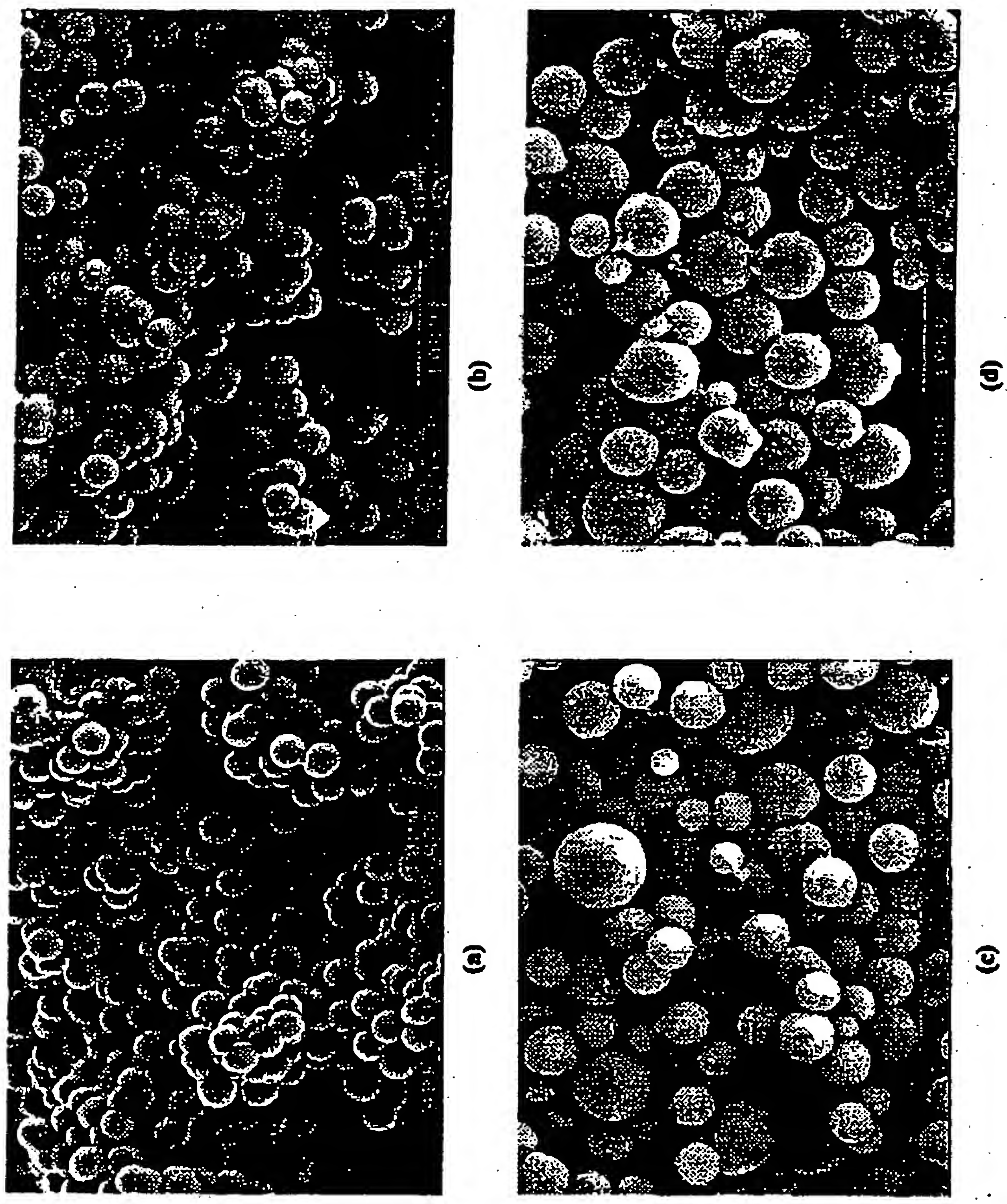


Figure 9

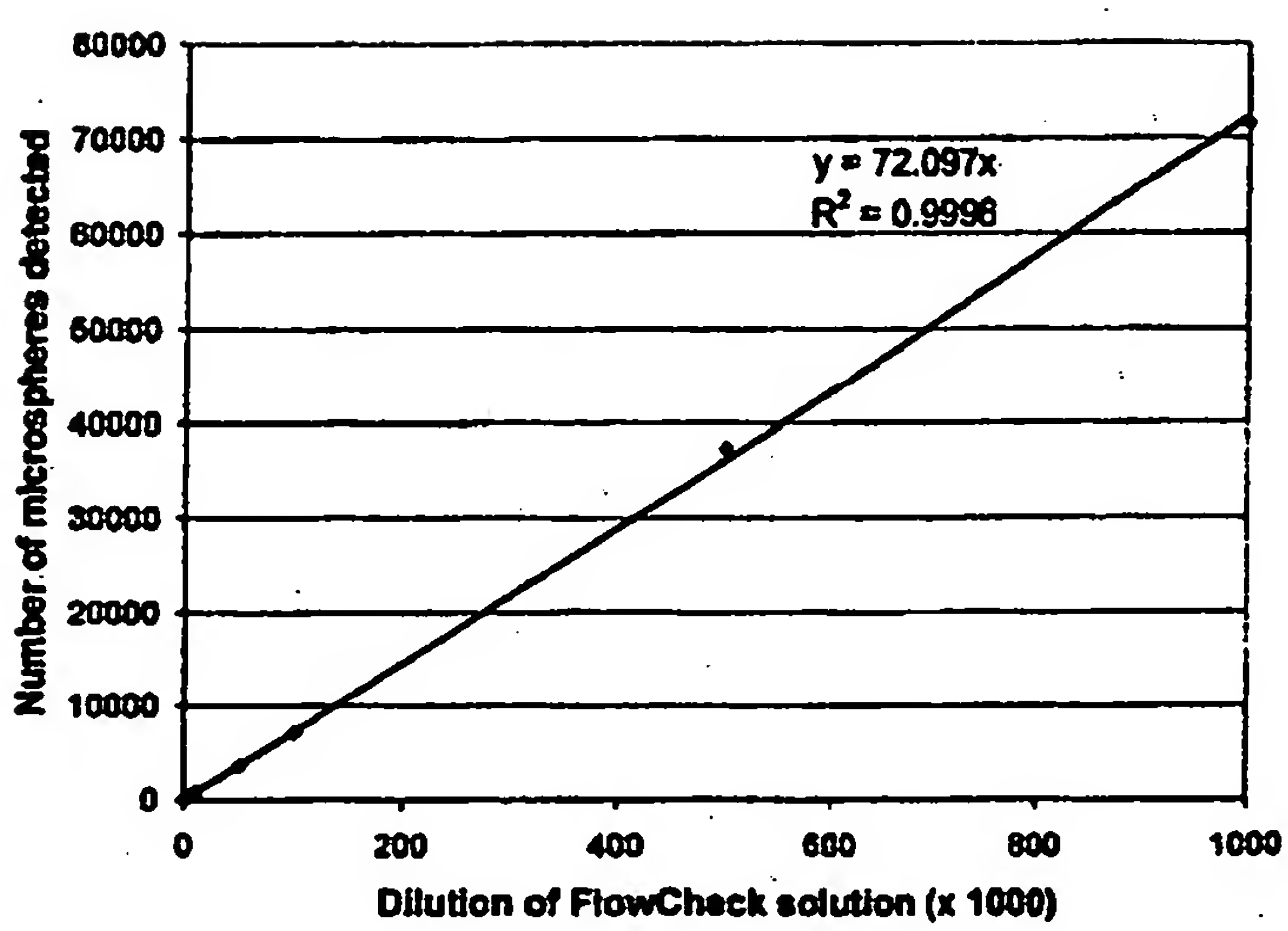

80000
70000
60000
50000
40000
30000
20000
10000
0
Number of microspheres detected
y = 72.097x
R² = 0.9998
0
200
400
600
800
1000
Dilution of FlowCheck solution (x 1000)

Figure 10

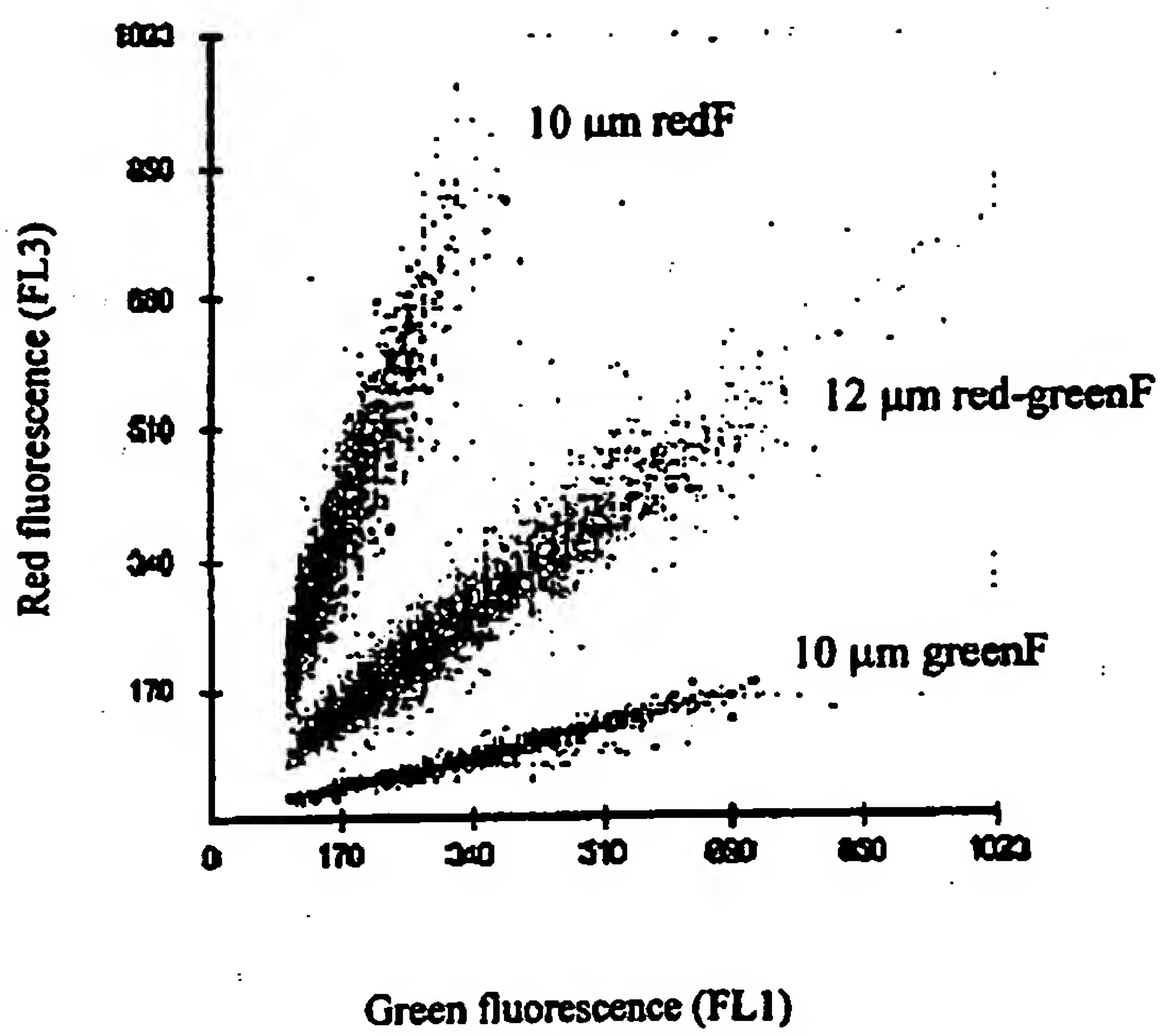

10 µm redF
12 µm red-greenF
10 µm greenF
Red fluorescence (FL3)
Green fluorescence (FL1)
0
170
340
510
680
850
1023

Figure 11

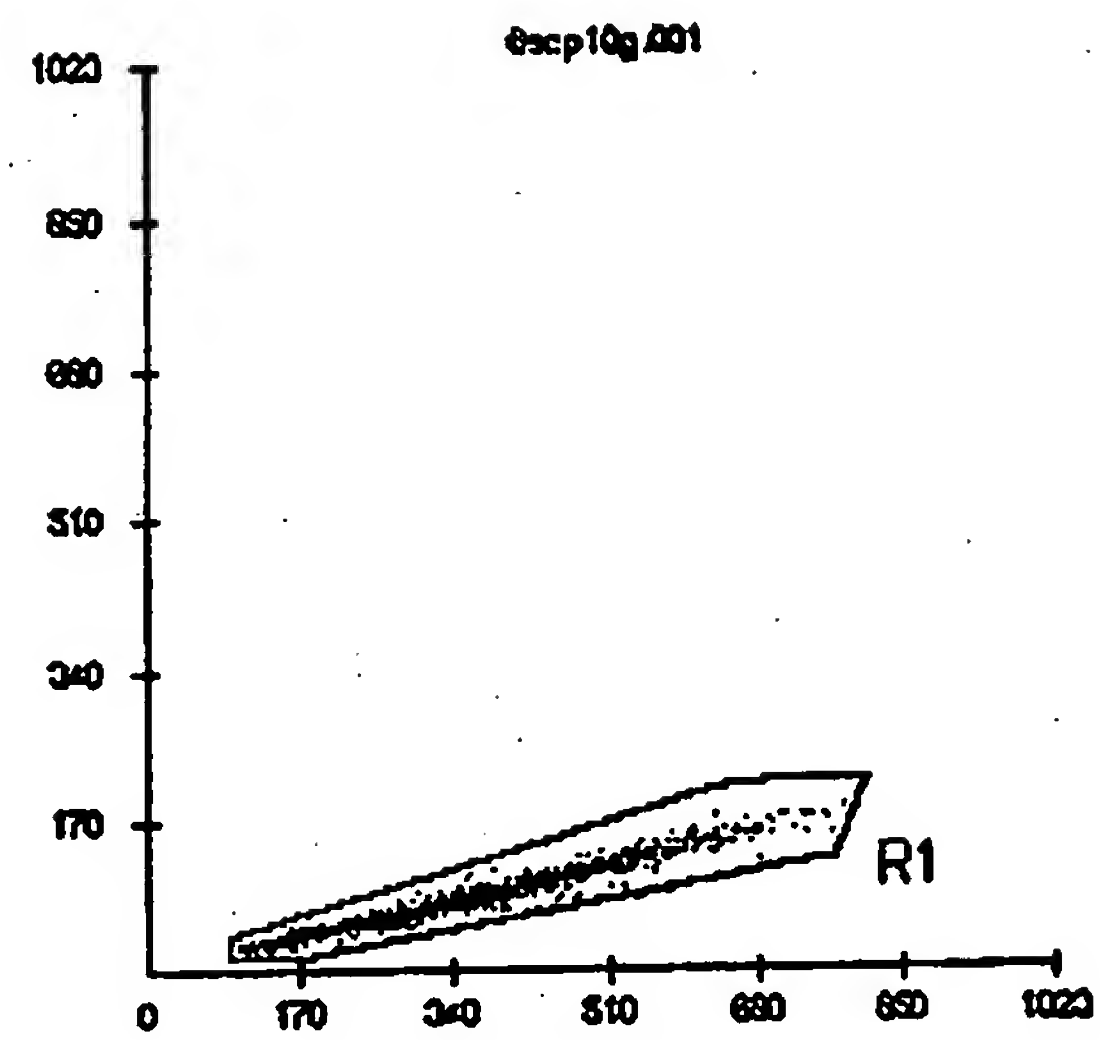

escp10g.001
1023
850
680
510
340
170
0
170
340
510
680
850
1023
R1

Figure 12

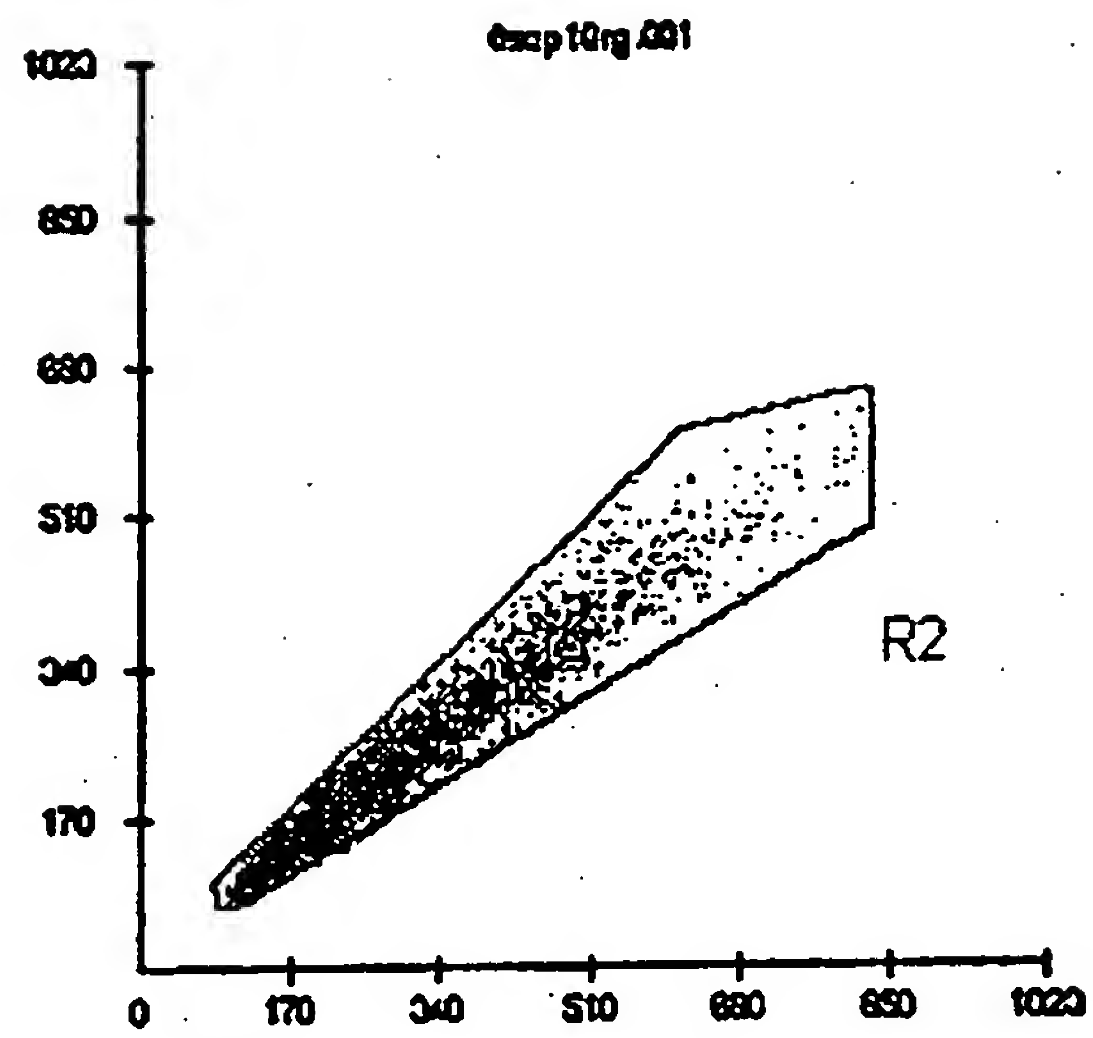


Figure 13

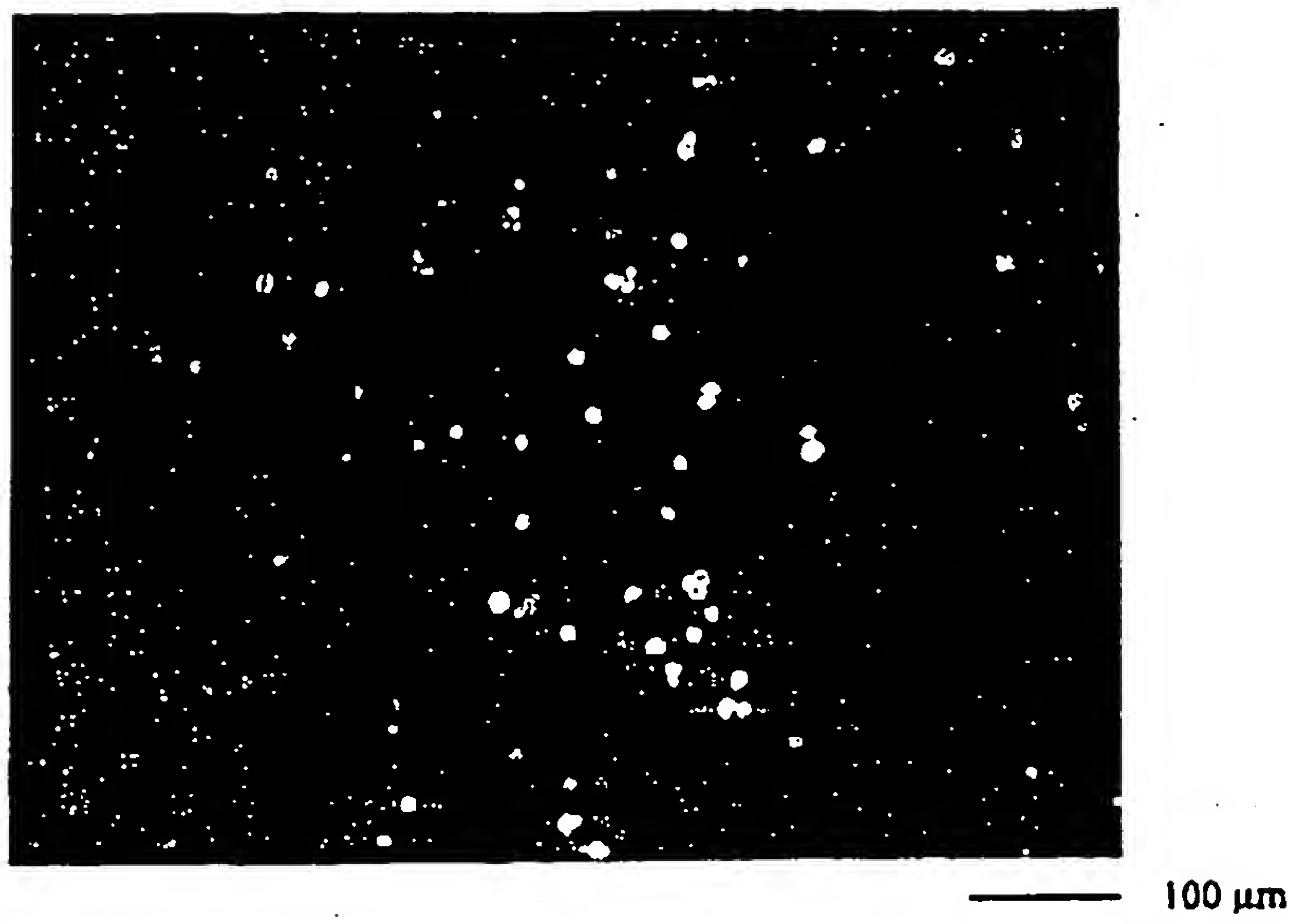


Figure 14

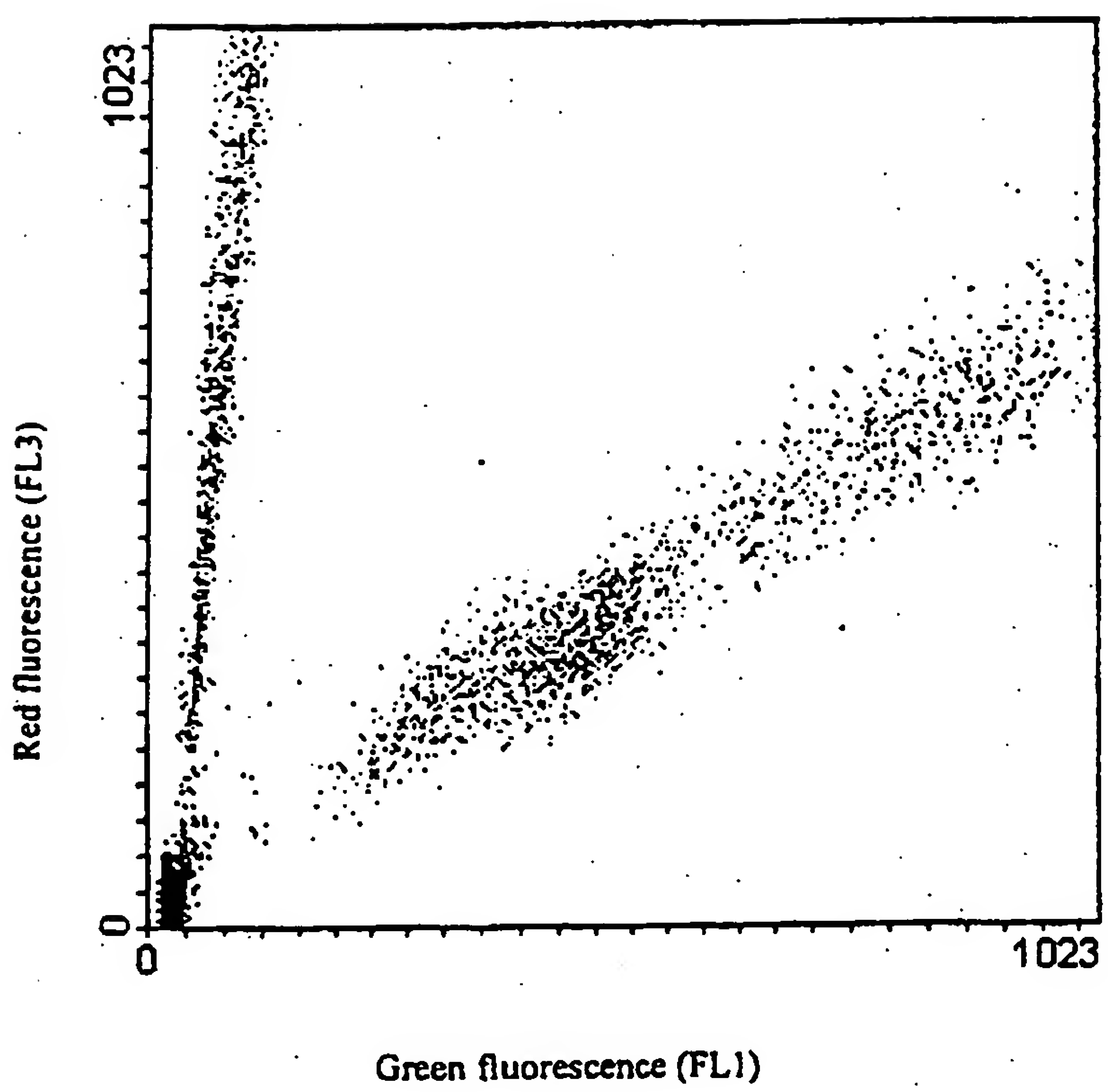


Figure 15

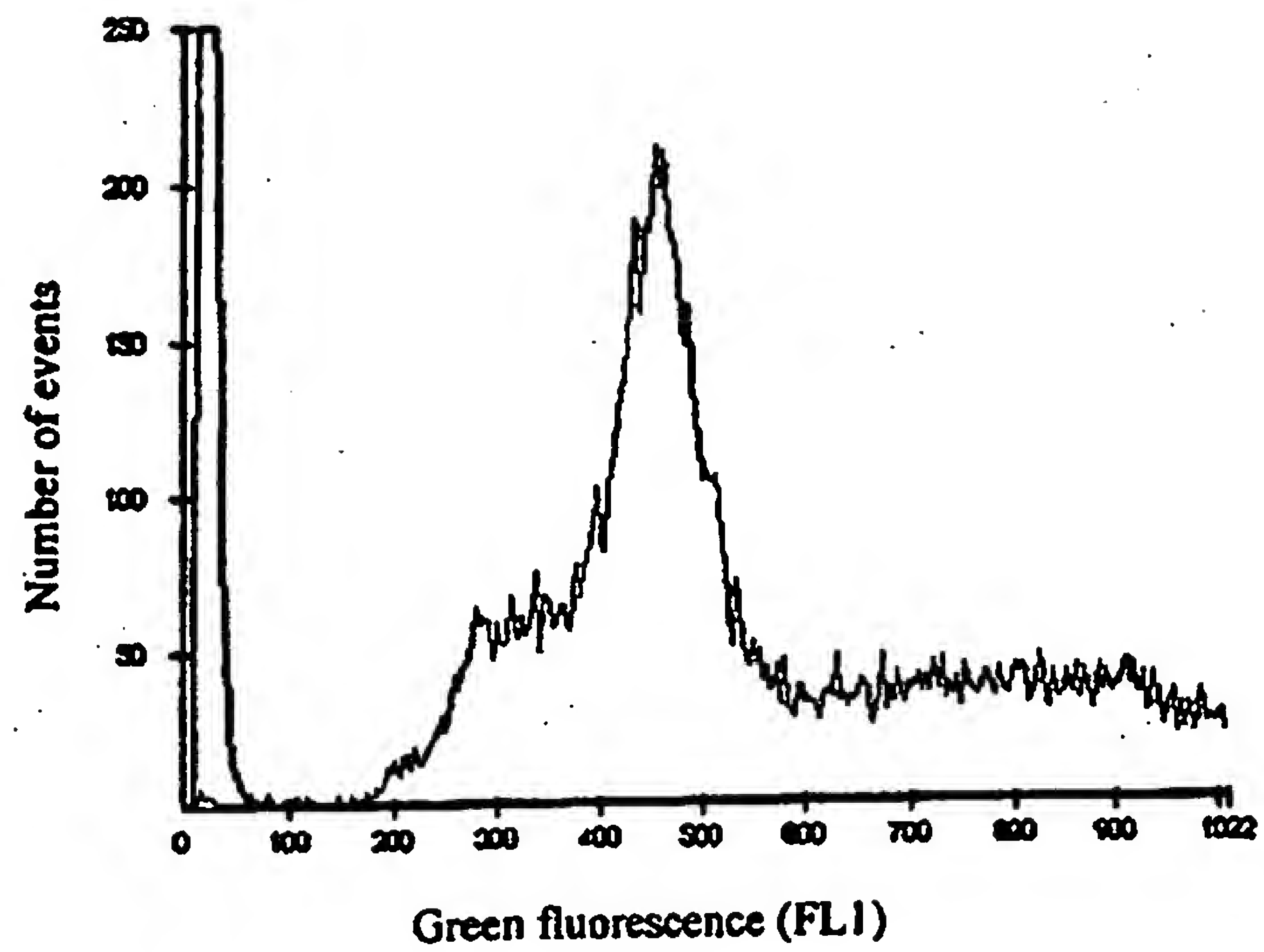


Figure 16(a)

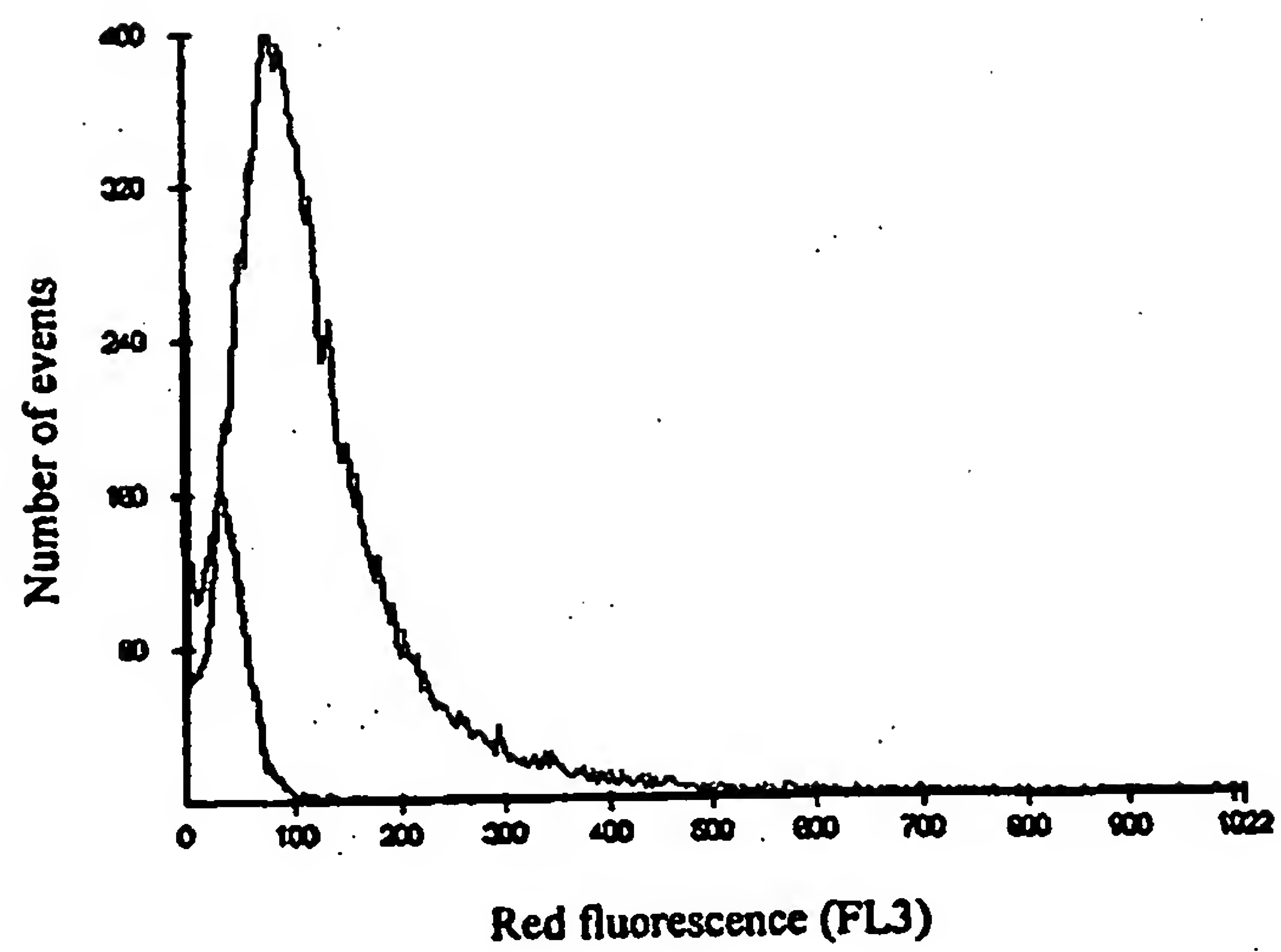


Figure 16(b)

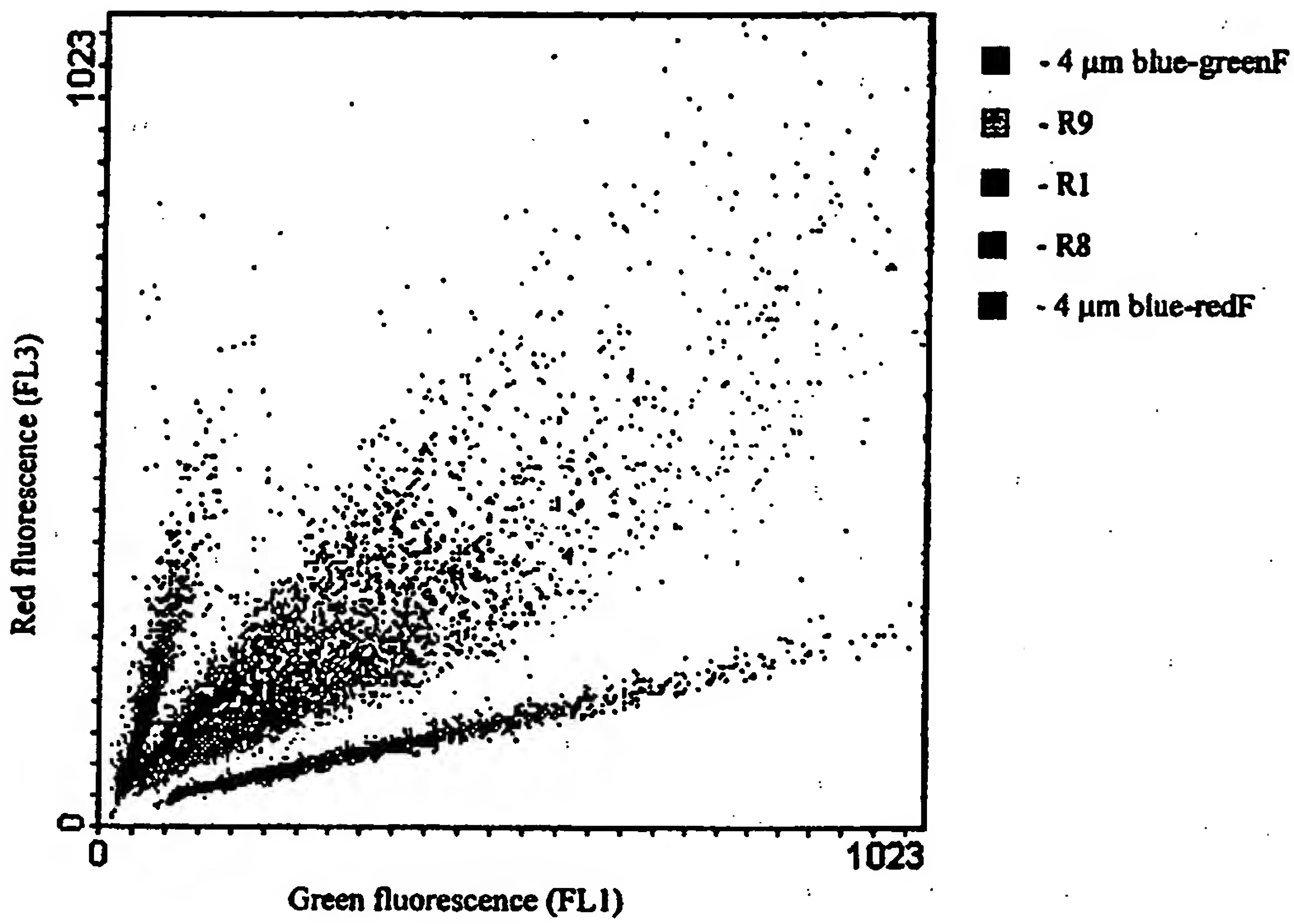

1023
0
Red fluorescence (FL3)
0
1023
Green fluorescence (FL1)
- 4 µm blue-greenF
- R9
- R1
- R8
- 4 µm blue-redF

Figure 17

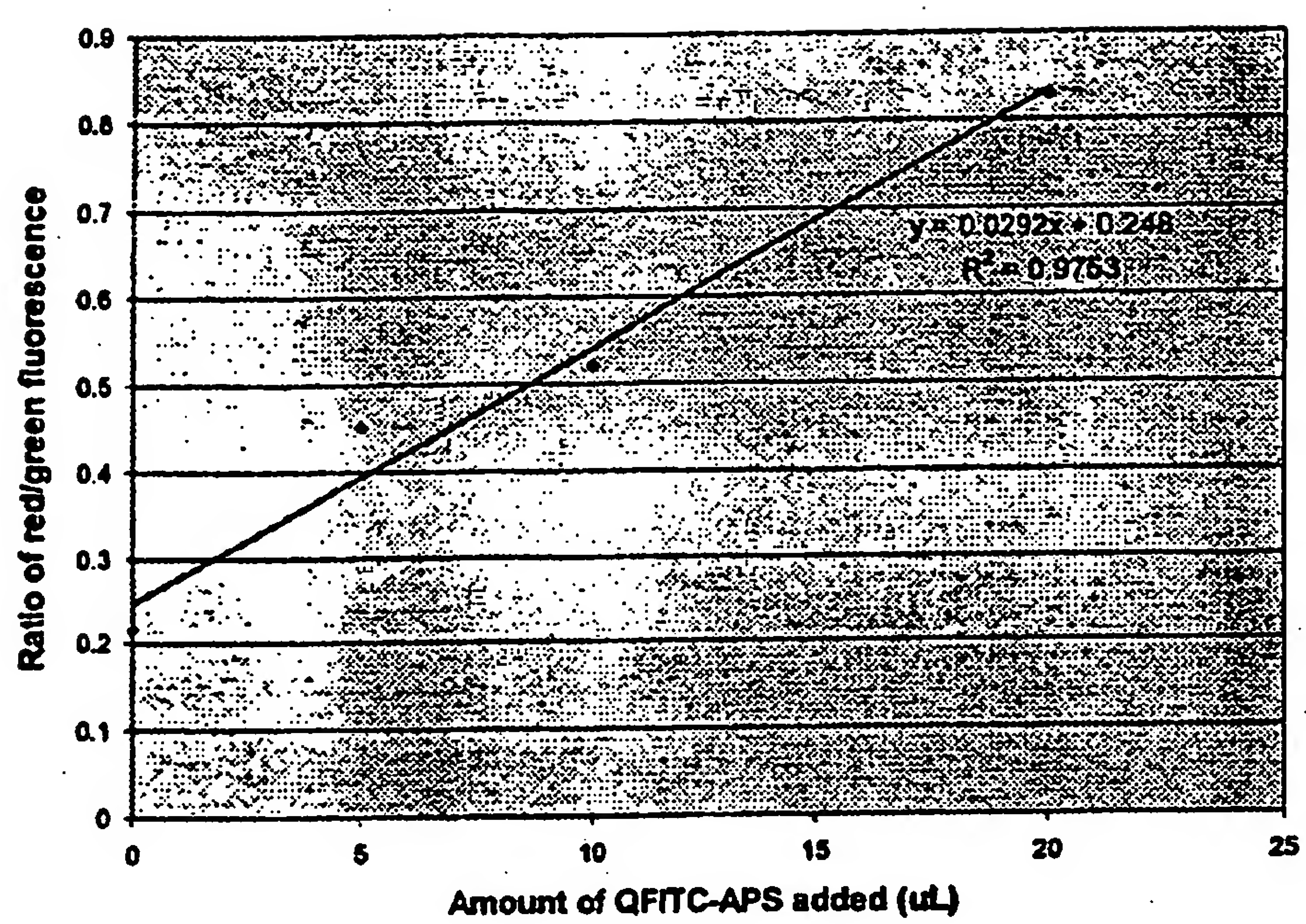
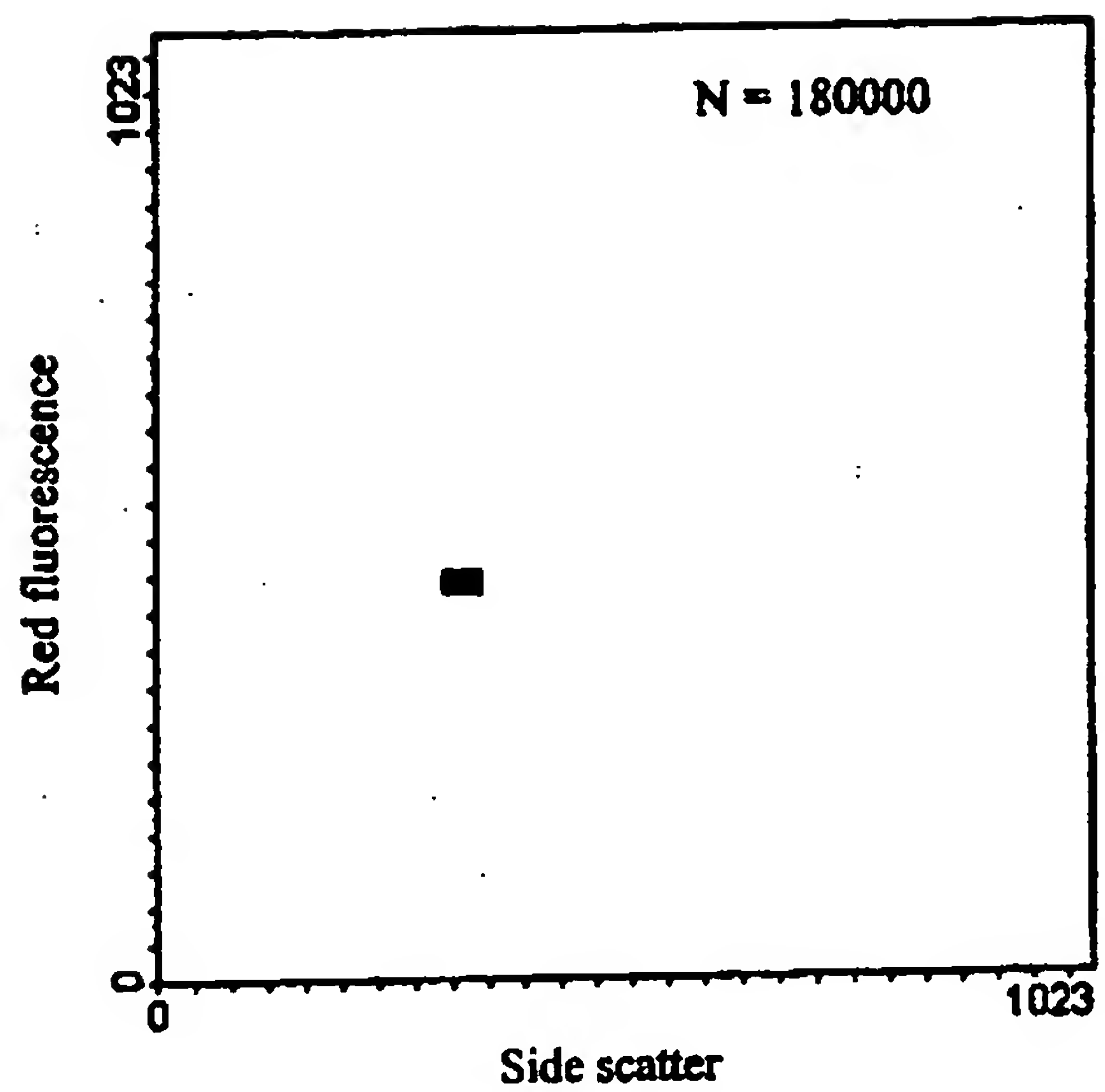

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Ratio of red/green fluorescence
y = 0.0292x + 0.248
R2 = 0.9753
0
5
10
15
20
25
Amount of QFITC-APS added (uL)

Figure 18


N = 180000
1023
Red fluorescence
0
0
1023
Side scatter

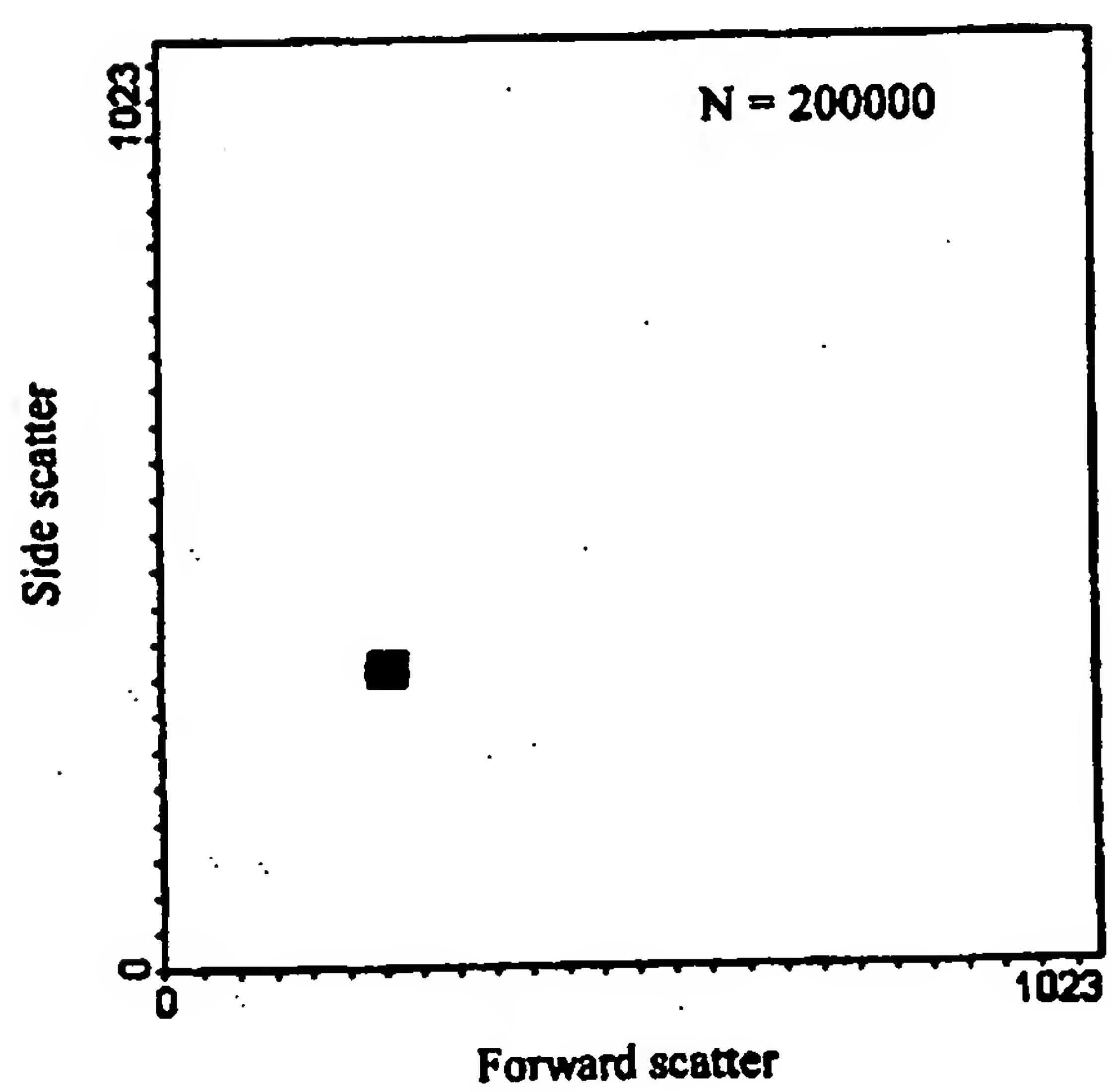

N = 200000
1023
Side scatter
0
0
1023
Forward scatter

Figure 19(a)

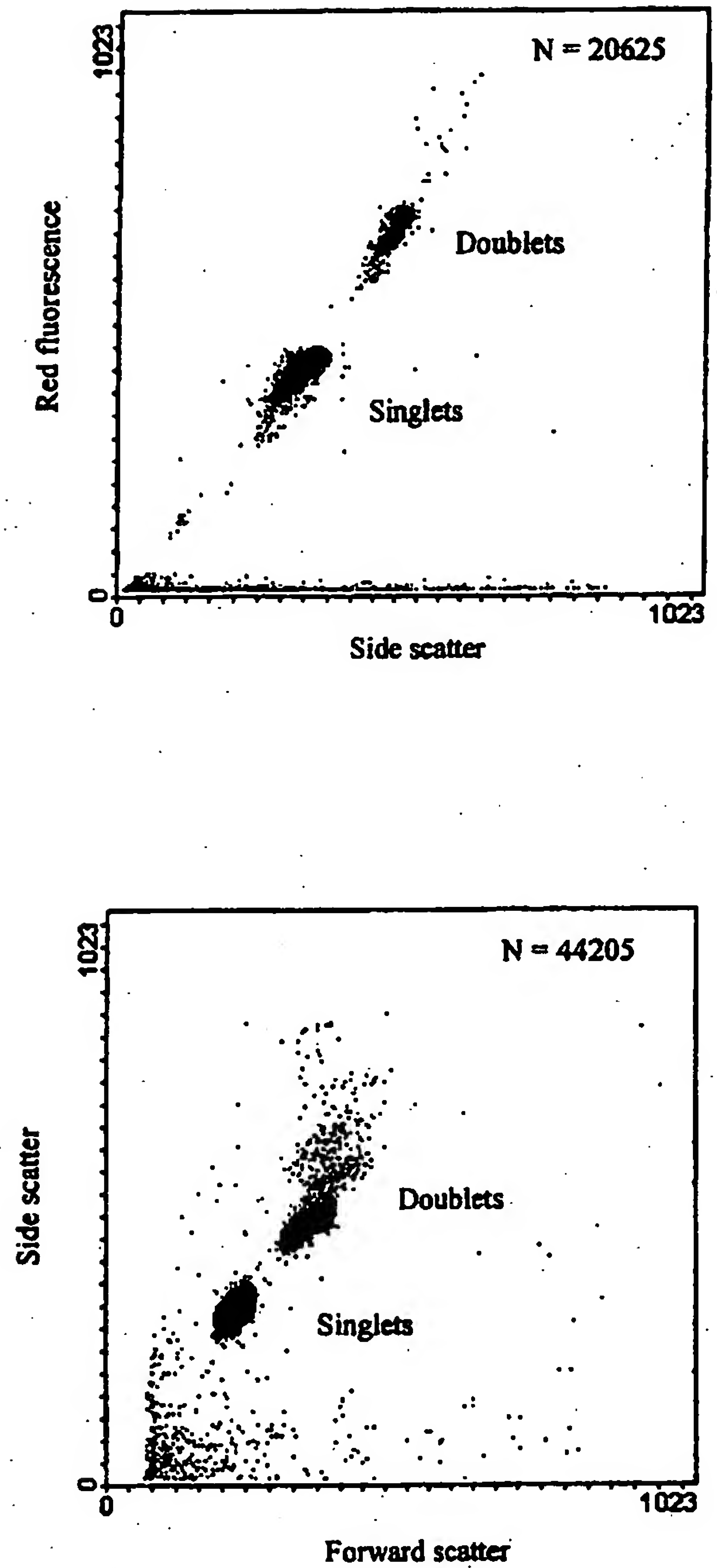
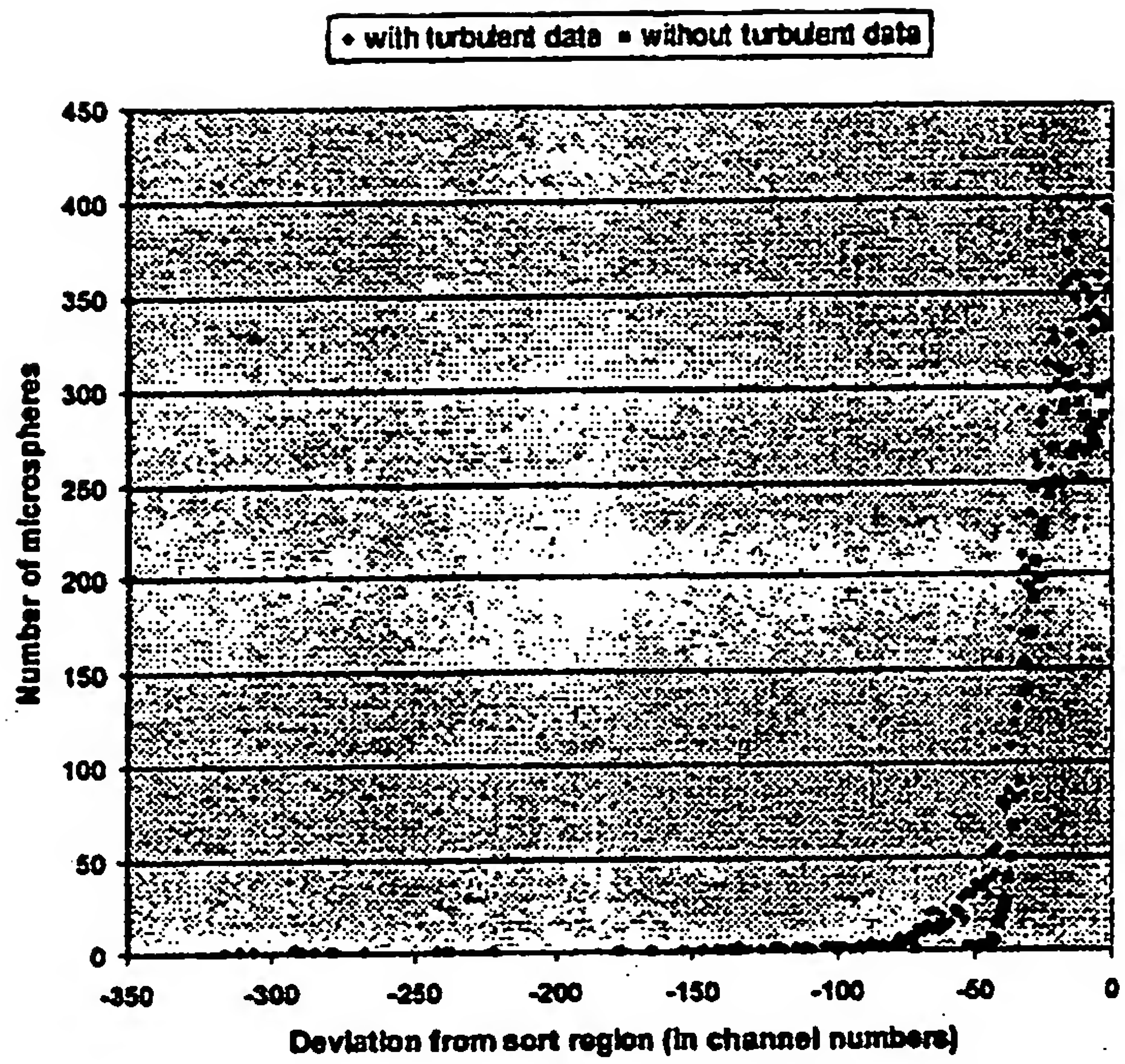

N = 20625
1023
Red fluorescence
0
Doublets
Singlets
0
1023
Side scatter
N = 44205
1023
Side scatter
0
Doublets
Singlets
0
1023
Forward scatter

Figure 19(b)



• with turbulent data ▪ without turbulent data
450
400
350
300
250
200
150
100
50
0
Number of microspheres
-350
-300
-250
-200
-150
-100
-50
0
Deviation from sort region (in channel numbers)

Figure 20

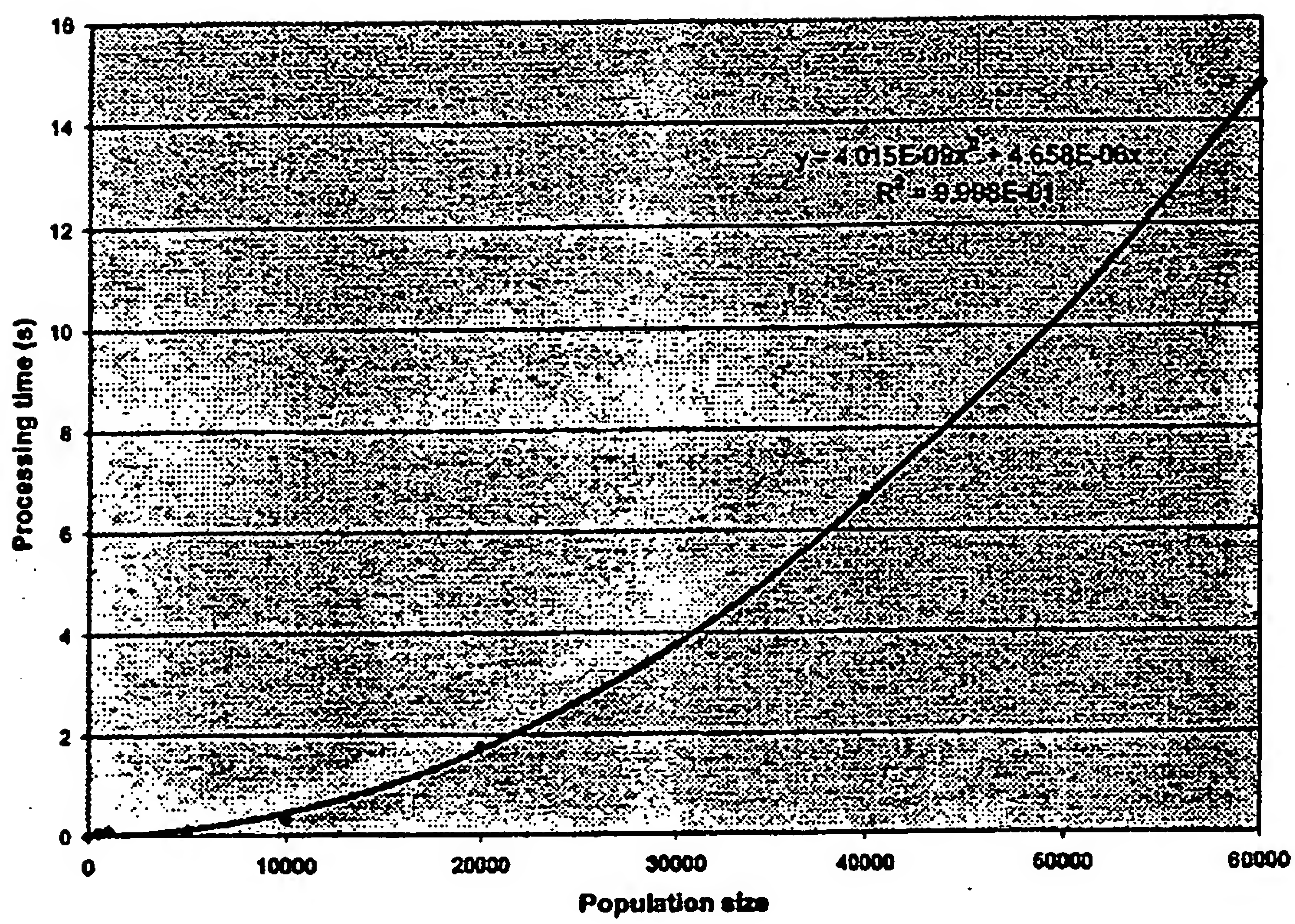
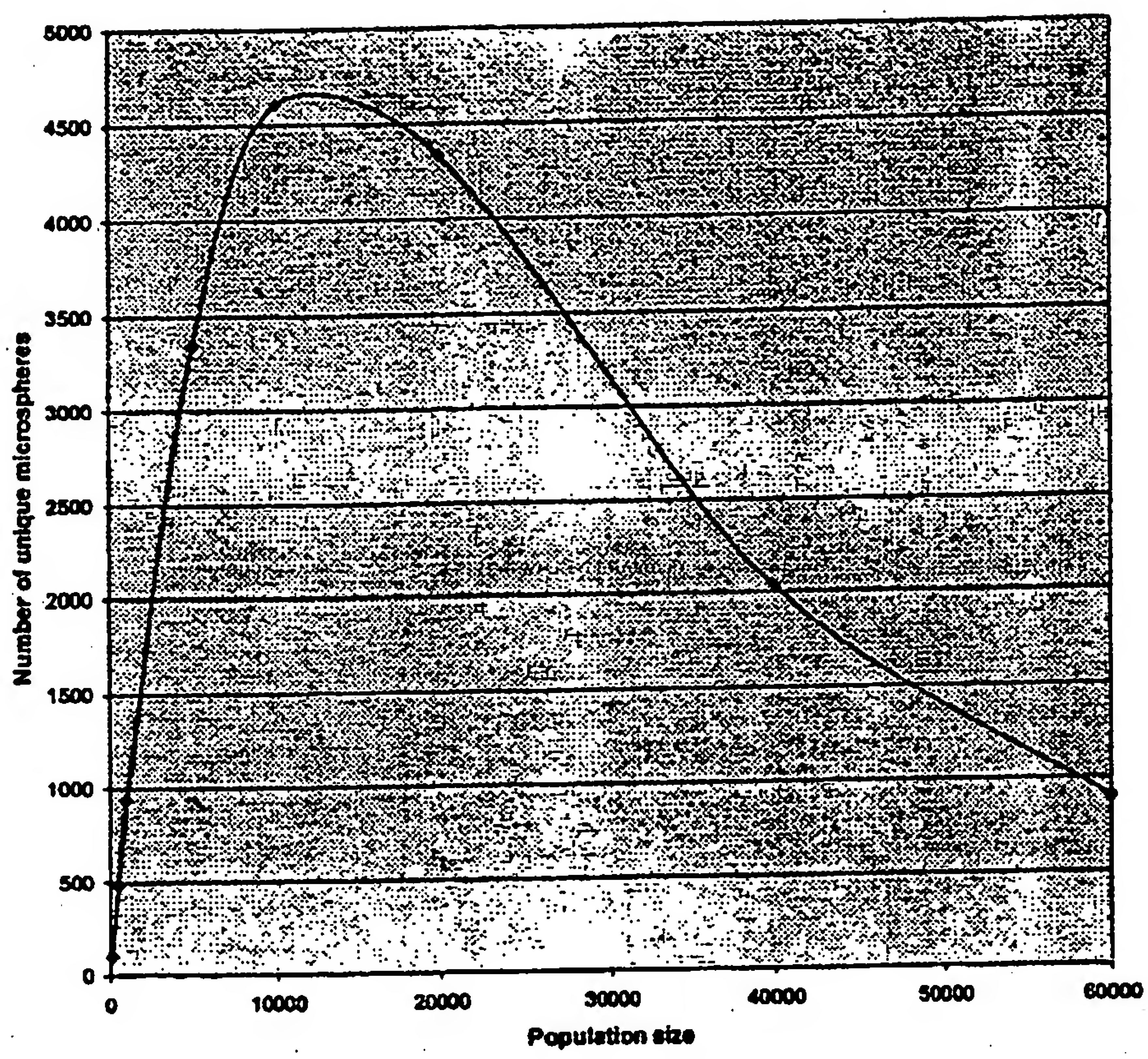
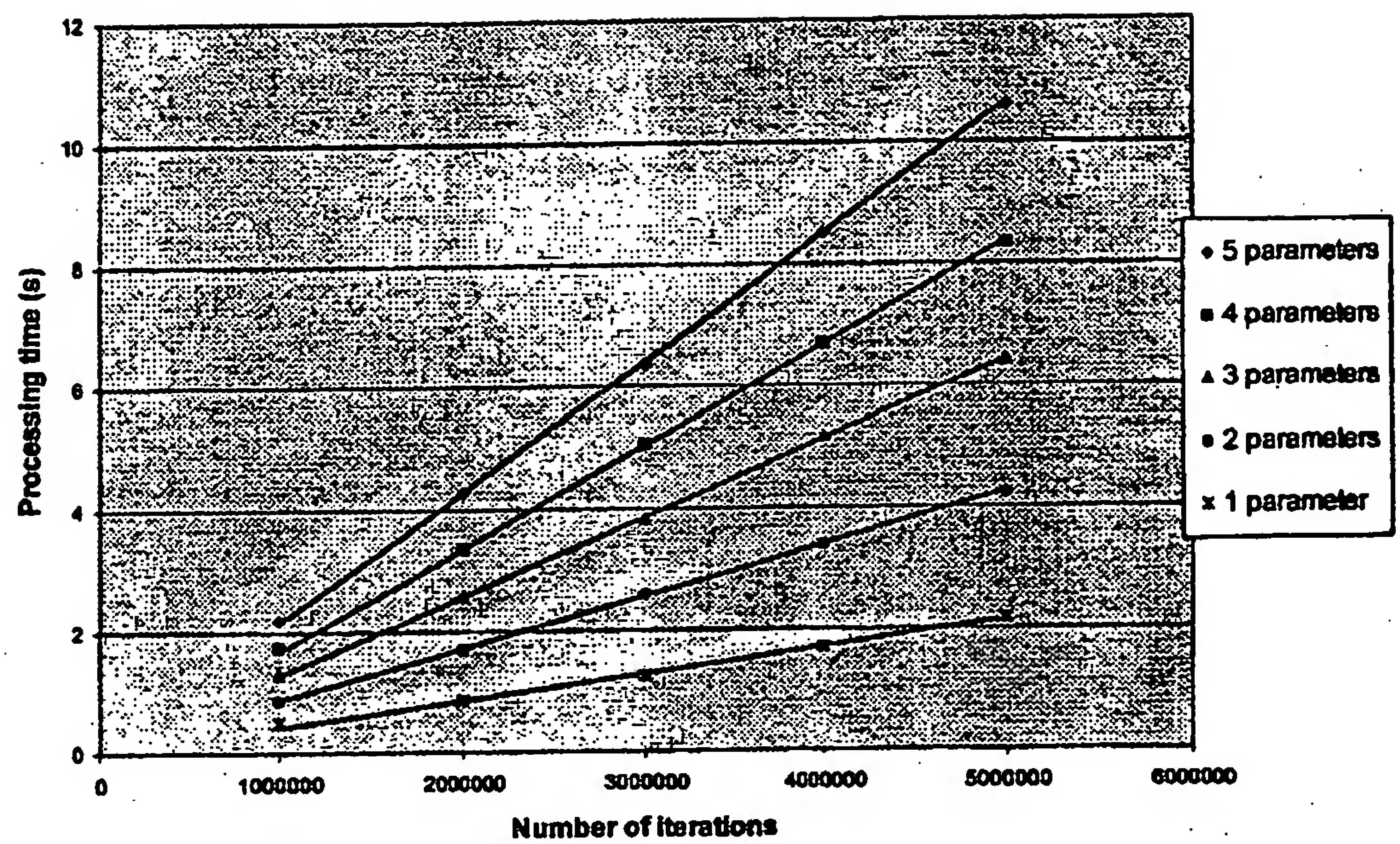

y = 4.015E-09x² + 4.658E-06x
R² = 9.998E-01
Processing time (s)
0
2
4
6
8
10
12
14
16
0
10000
20000
30000
40000
50000
60000
Population size

Figure 21



5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Number of unique microspheres
0
10000
20000
30000
40000
50000
60000
Population size

Figure 22



12
10
8
6
4
2
0
Processing time (s)
0
1000000
2000000
3000000
4000000
5000000
6000000
Number of iterations
5 parameters
4 parameters
3 parameters
2 parameters
1 parameter

Figure 23

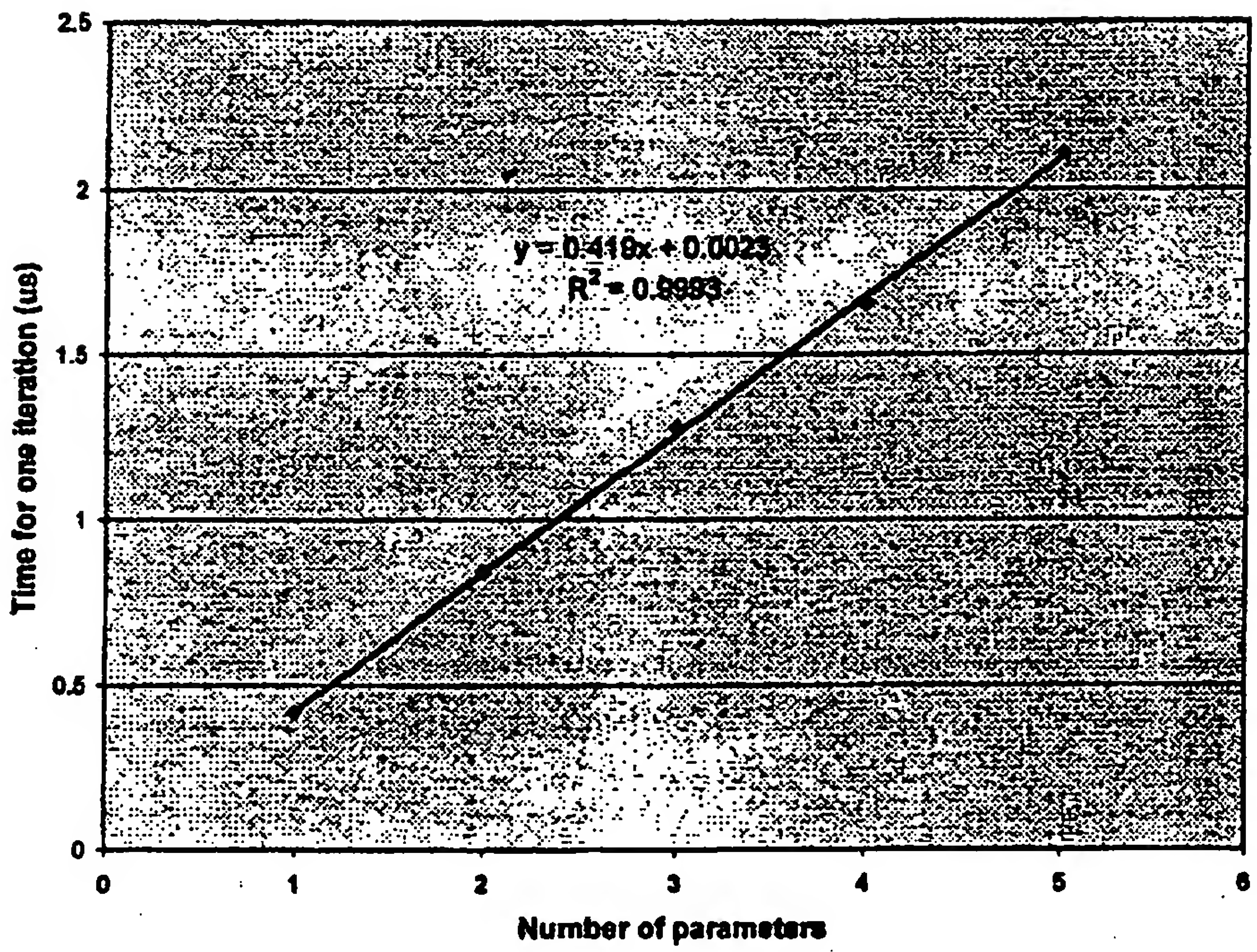
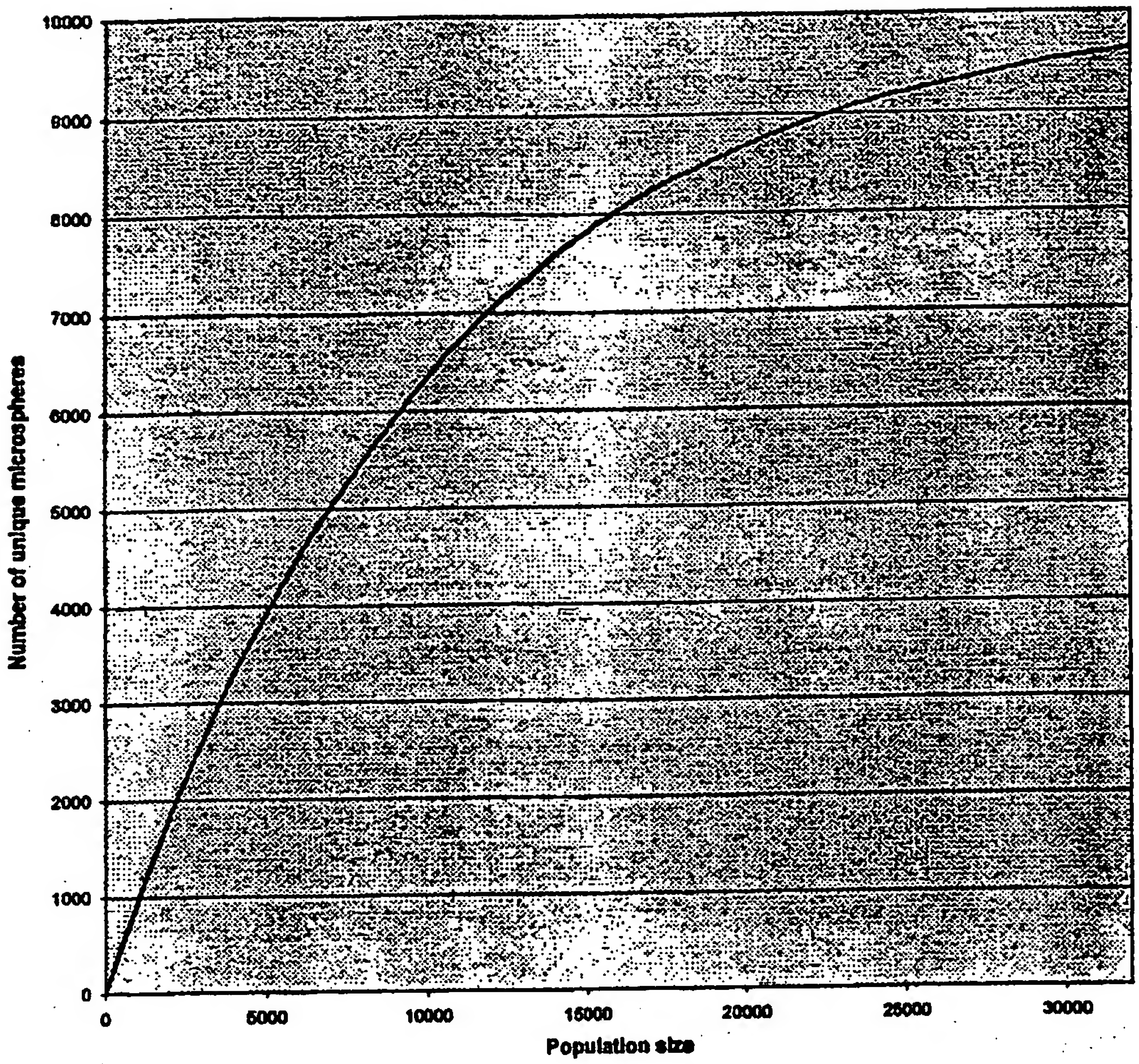

2.5
2
1.5
1
0.5
0
0 1 2 3 4 5 6
Time for one iteration (us)
Number of parameters
y = 0.419x + 0.0023
R^2 = 0.9993

Figure 24



10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Number of unique microspheres
0
5000
10000
15000
20000
25000
30000
Population size

Figure 25

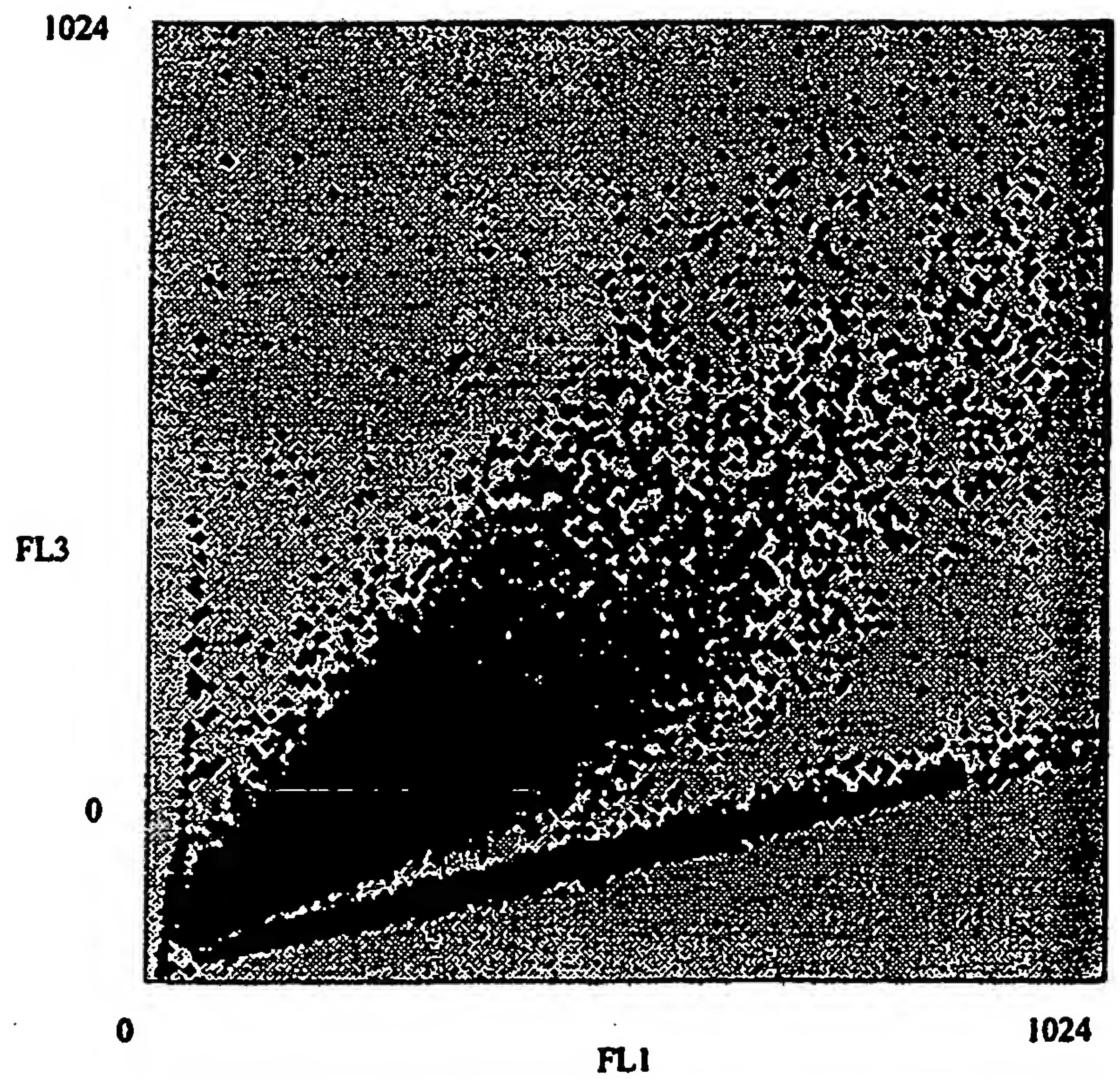


Figure 26

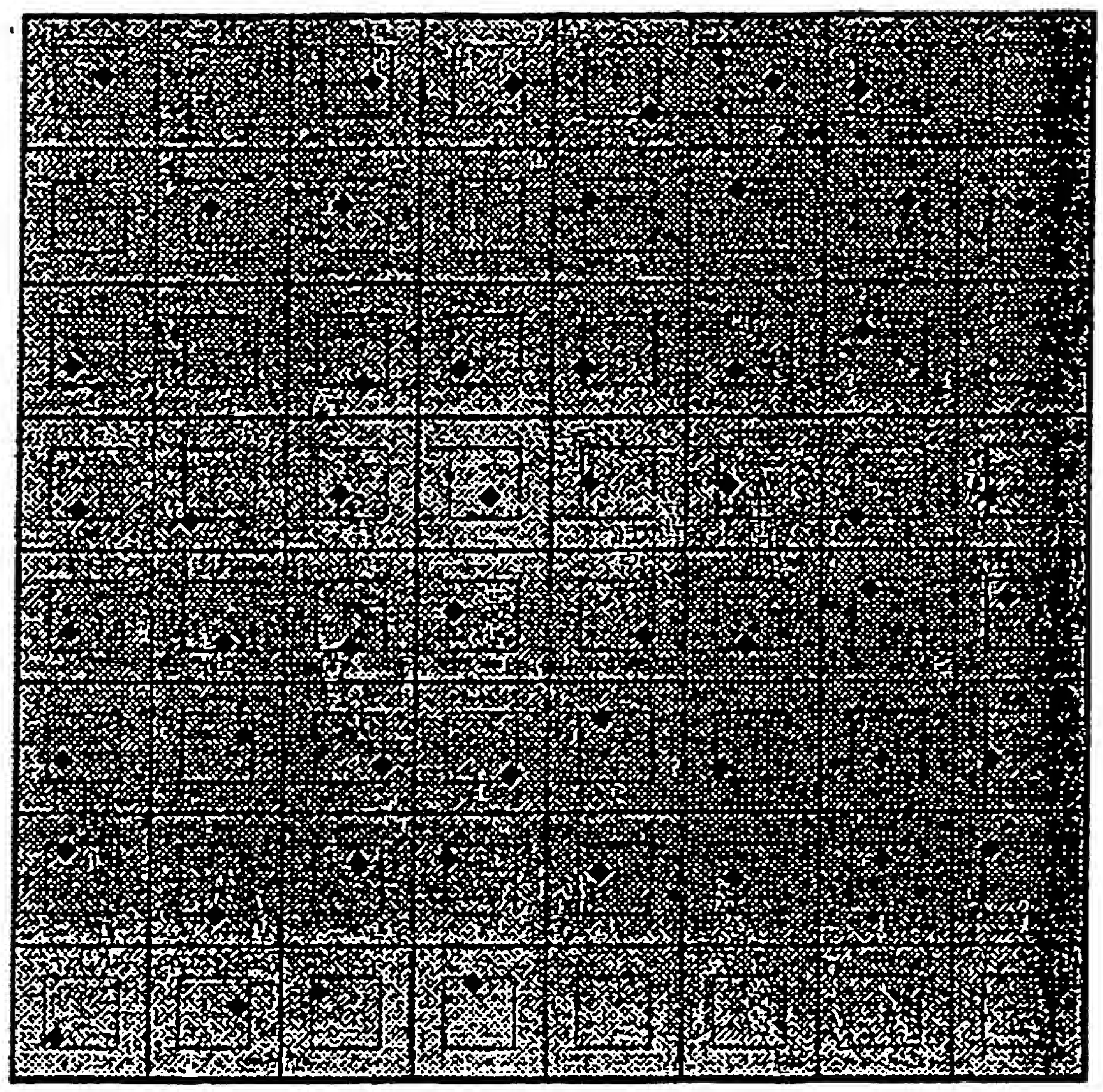
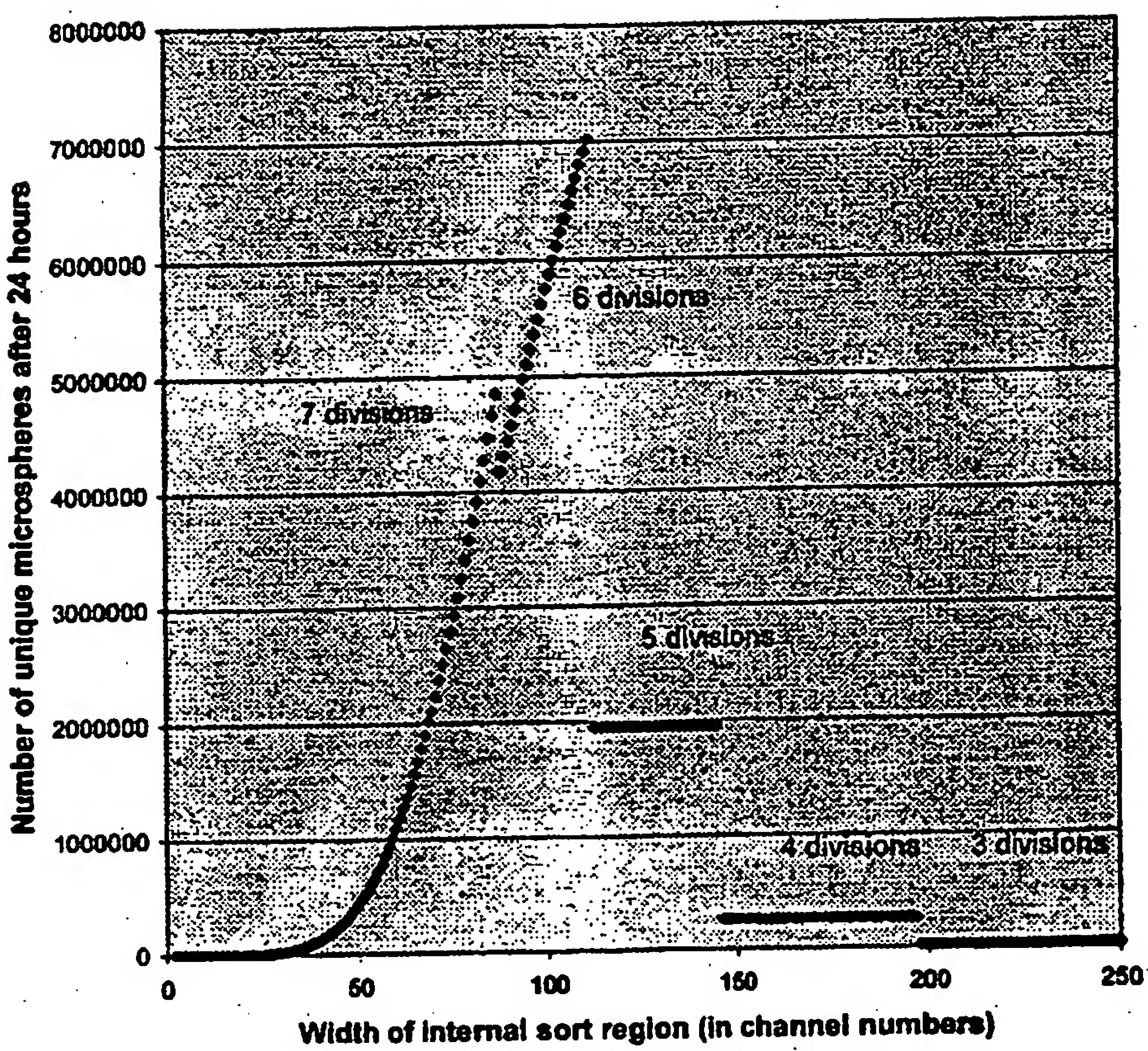
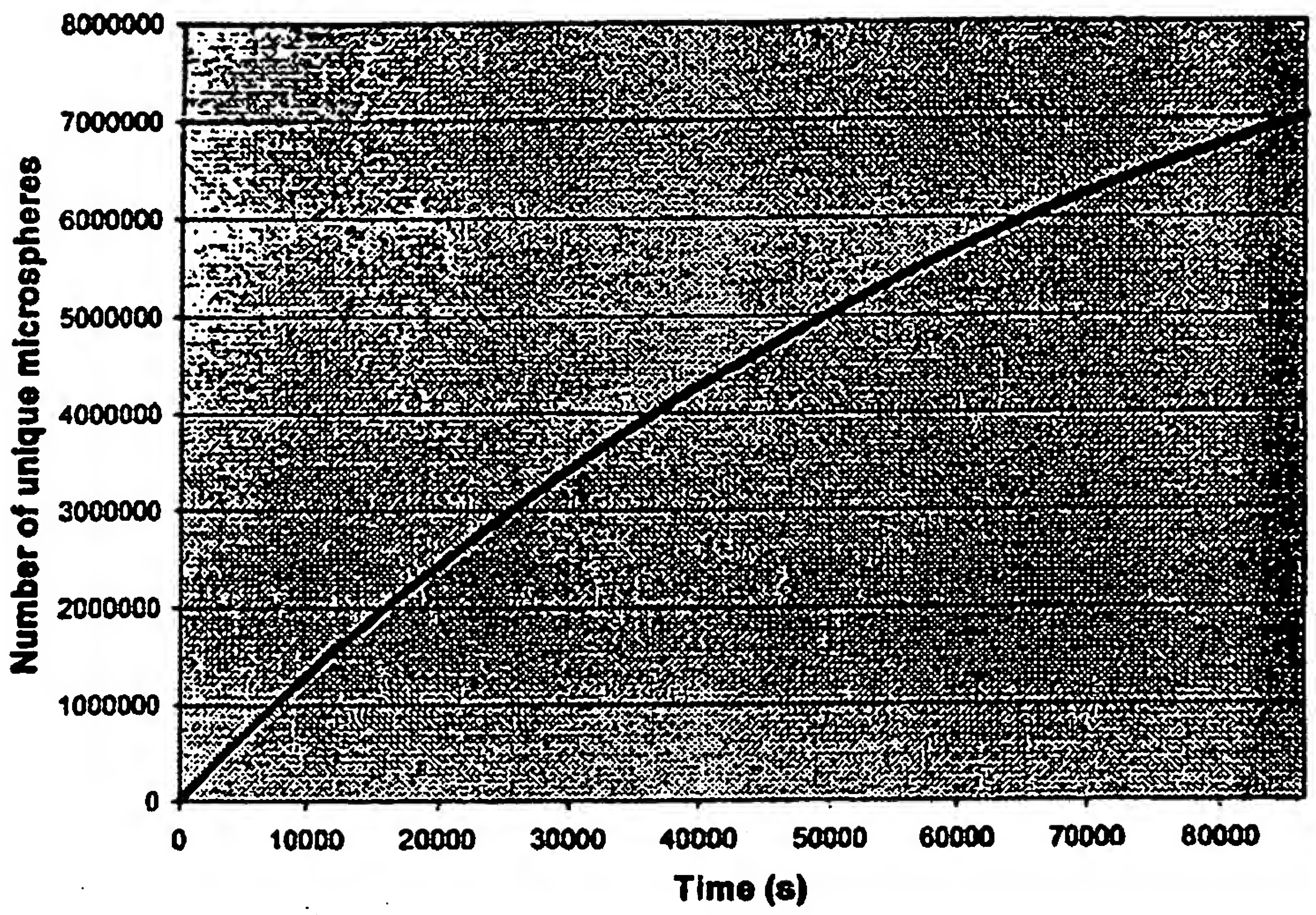


Figure 27



8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
Number of unique microspheres after 24 hours
6 divisions
7 divisions
5 divisions
4 divisions
3 divisions
0
50
100
150
200
250
Width of internal sort region (in channel numbers)

Figure 28



8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
Number of unique microspheres
0
10000
20000
30000
40000
50000
60000
70000
80000
Time (s)

Figure 29

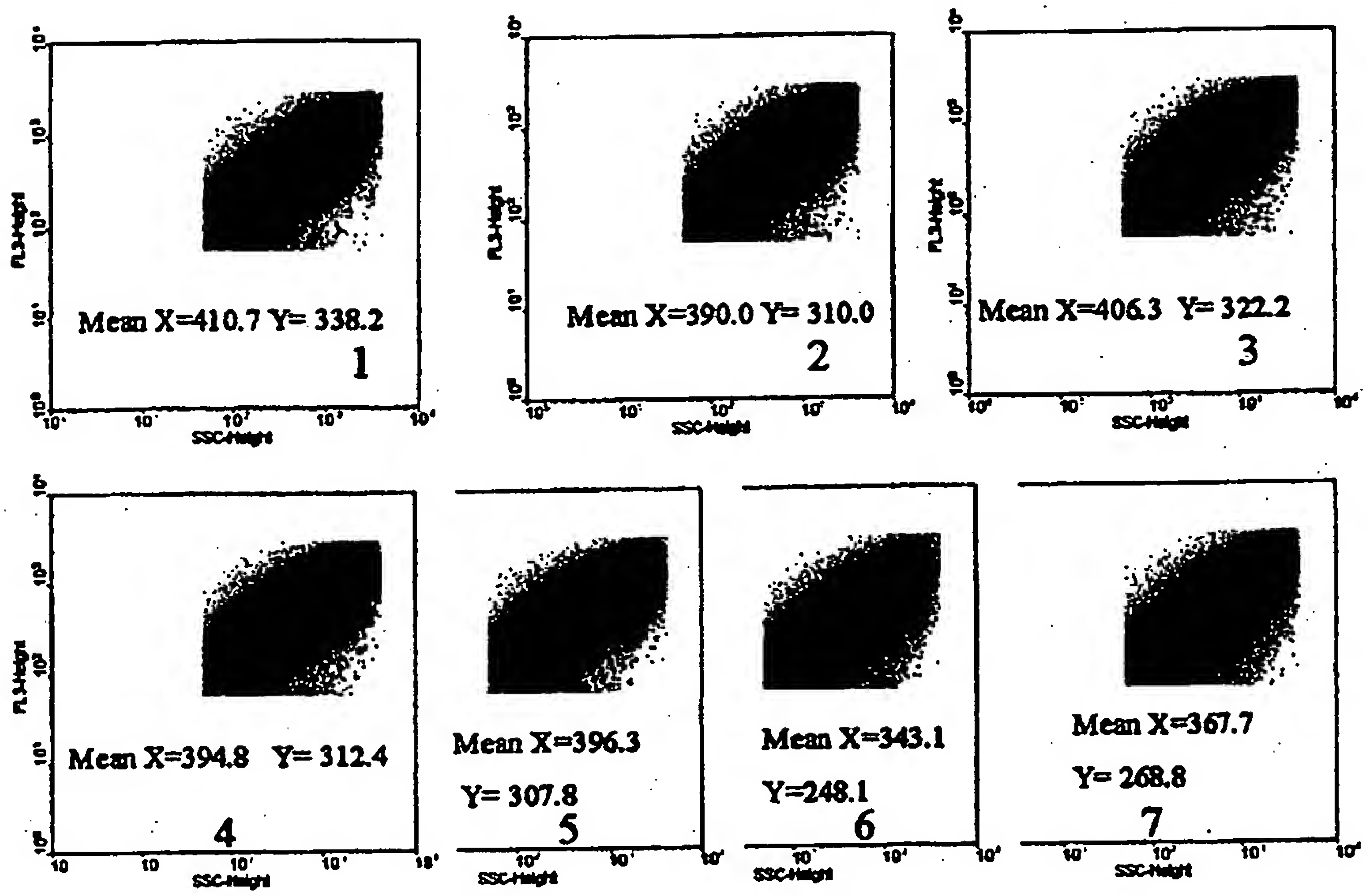
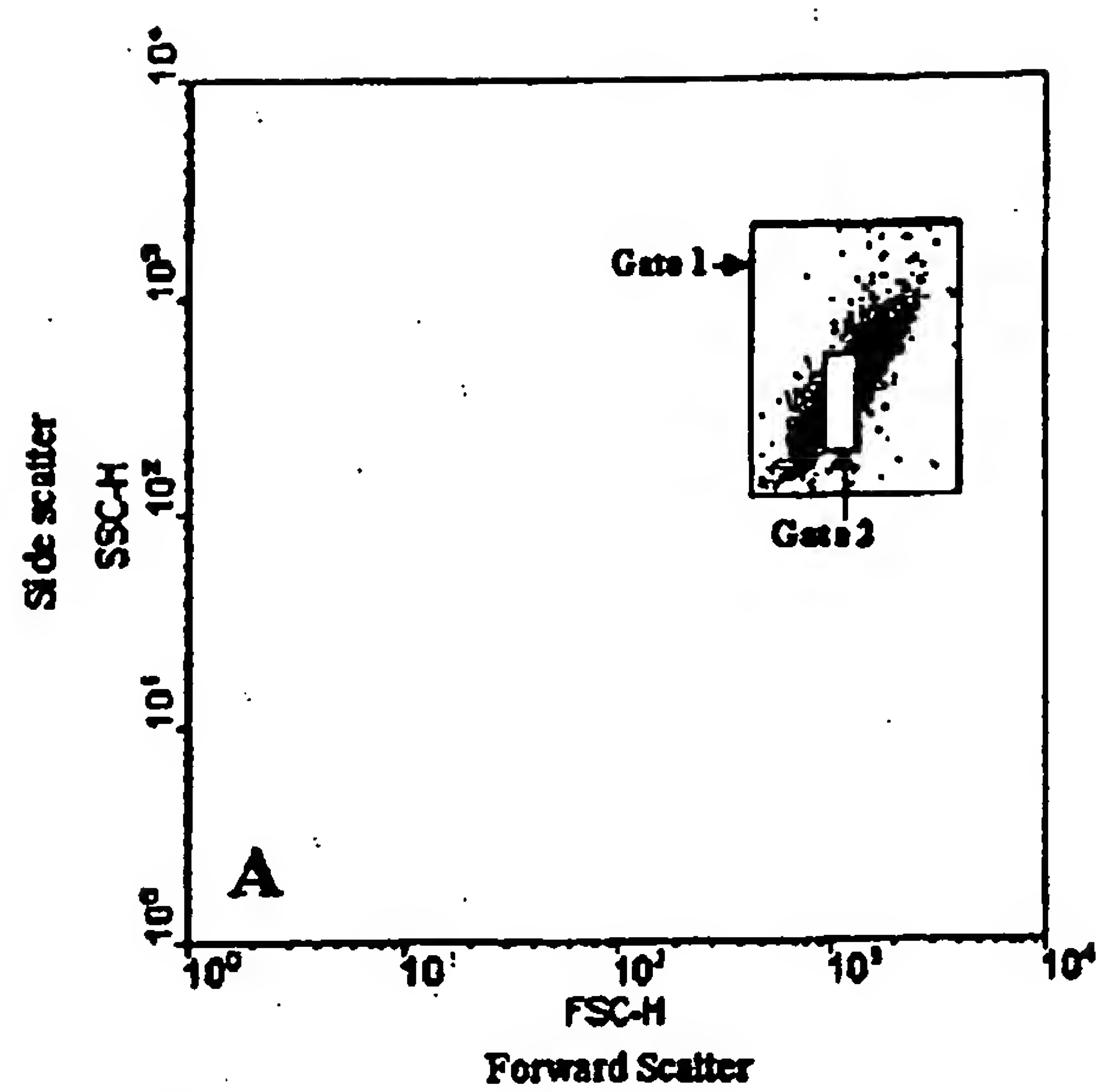

Mean X=410.7 Y= 338.2
1
Mean X=390.0 Y= 310.0
2
Mean X=406.3 Y= 322.2
3
Mean X=394.8 Y= 312.4
4
Mean X=396.3
Y= 307.8
5
Mean X=343.1
Y=248.1
6
Mean X=367.7
Y= 268.8
7
SSC-Height
FL3-Height

Figure 30


Side scatter
SSC-H
Gate 1
Gate 2
A
FSC-H
Forward Scatter

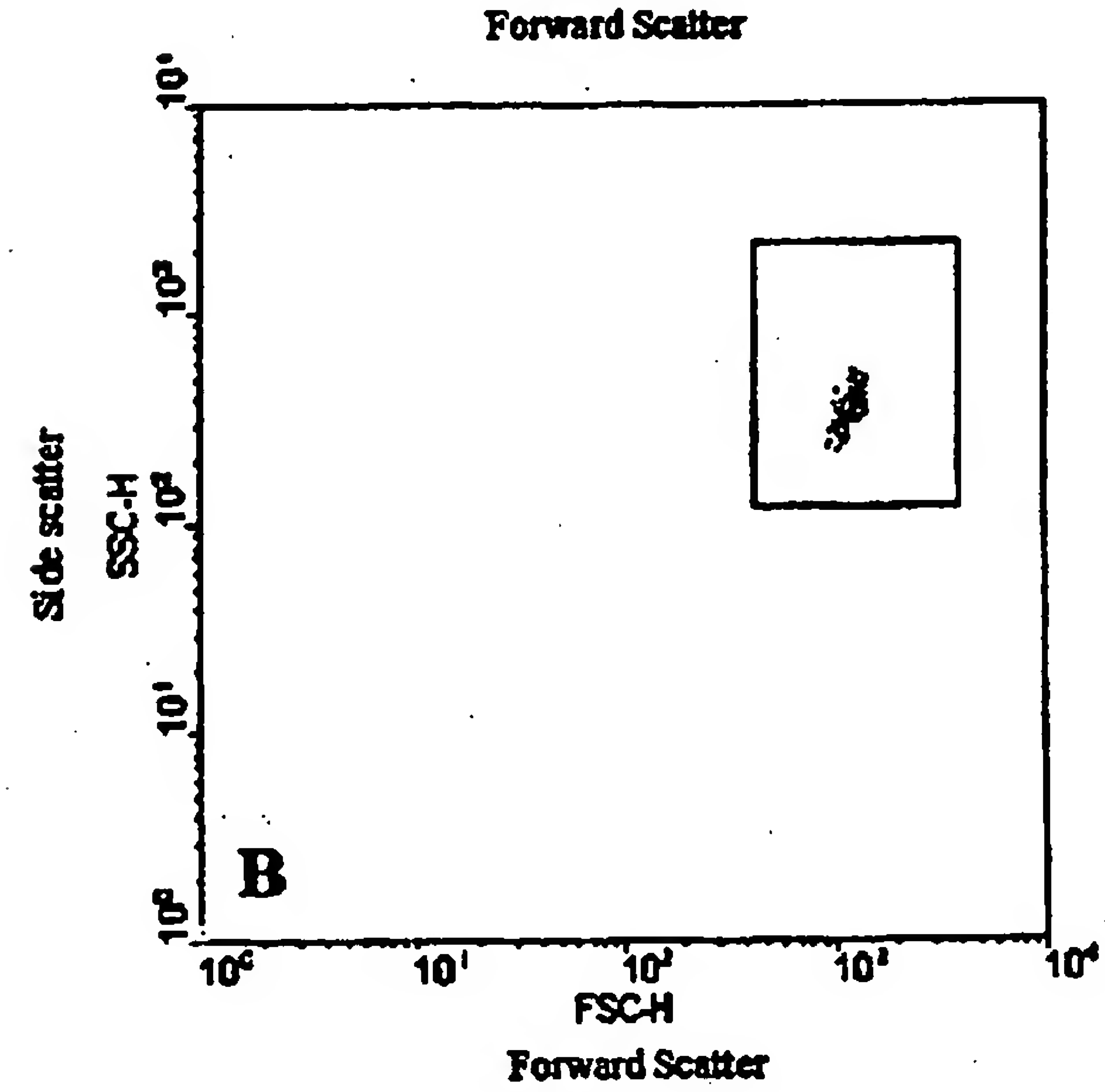
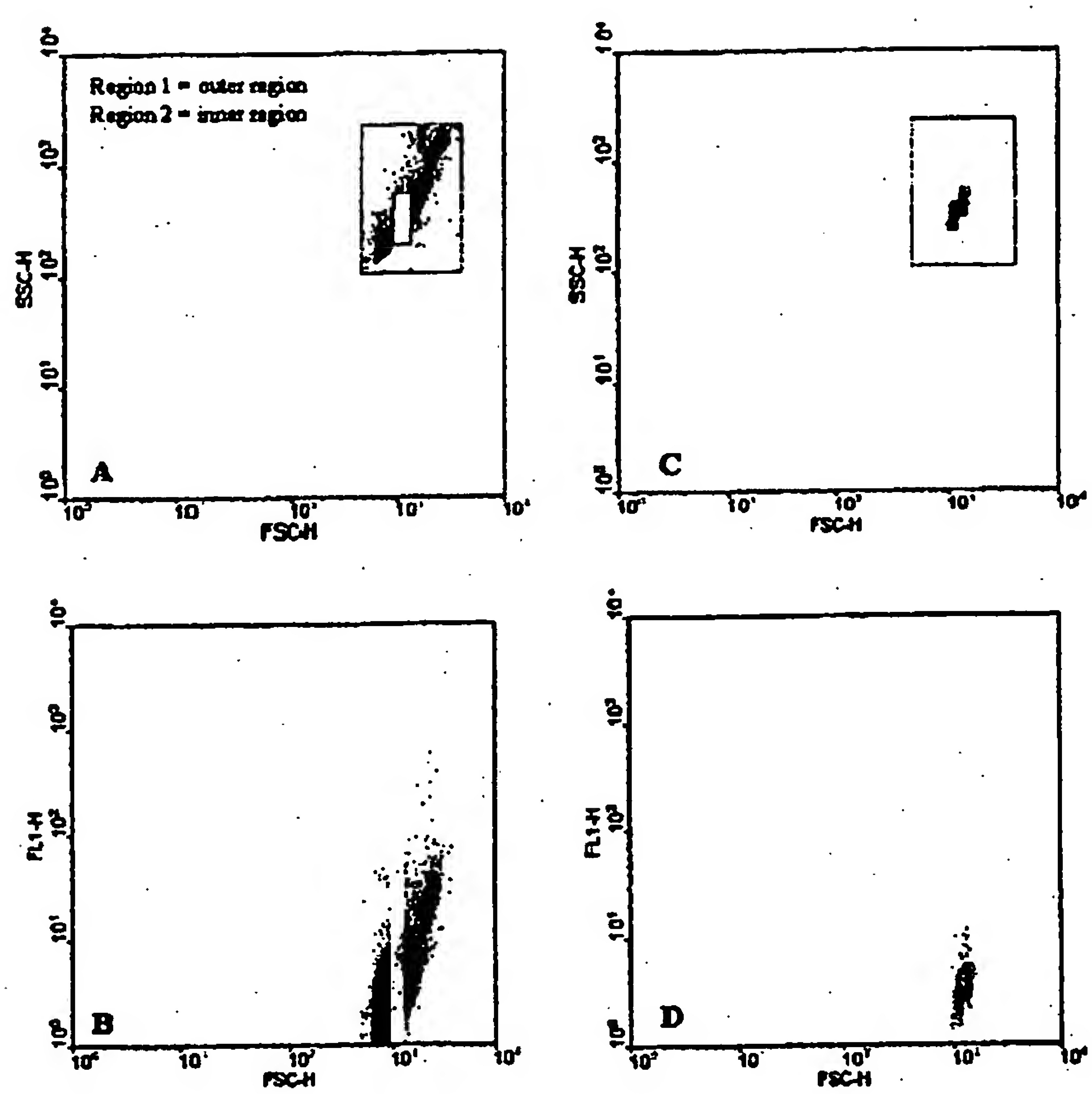

Side scatter
SSC-H
B
FSC-H
Forward Scatter

Figure 31



Region 1 = outer region
Region 2 = inner region
A
B
C
D
SSC-H
FL1-H
FSC-H

Figure 32

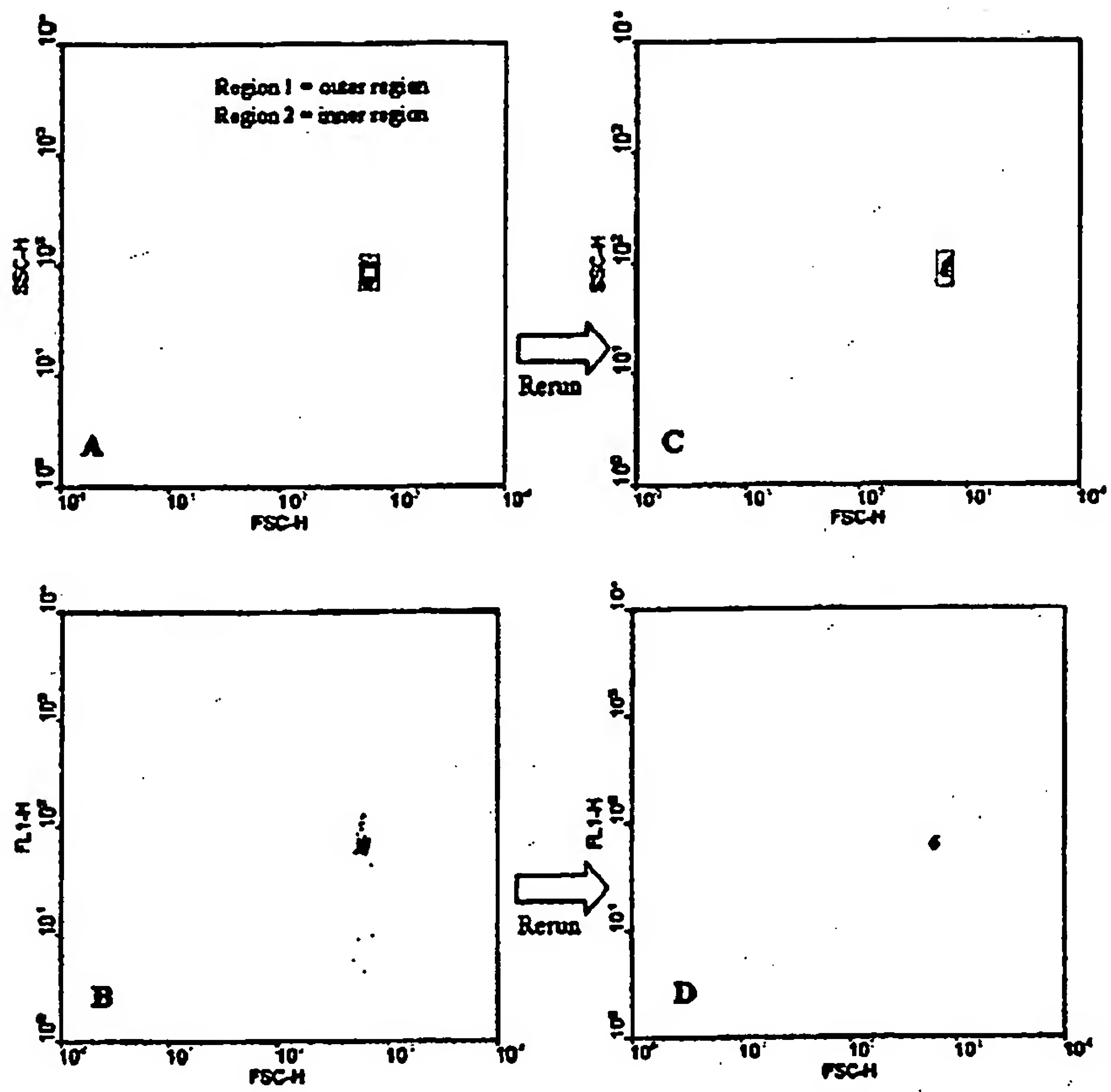
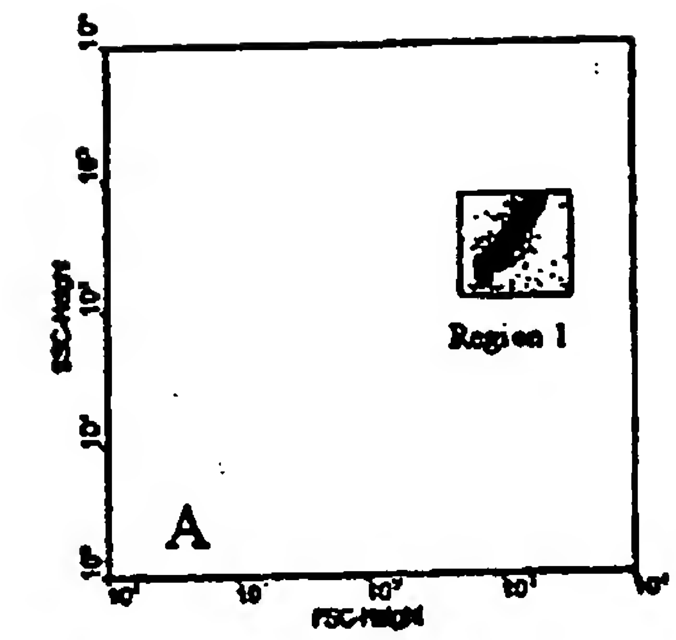
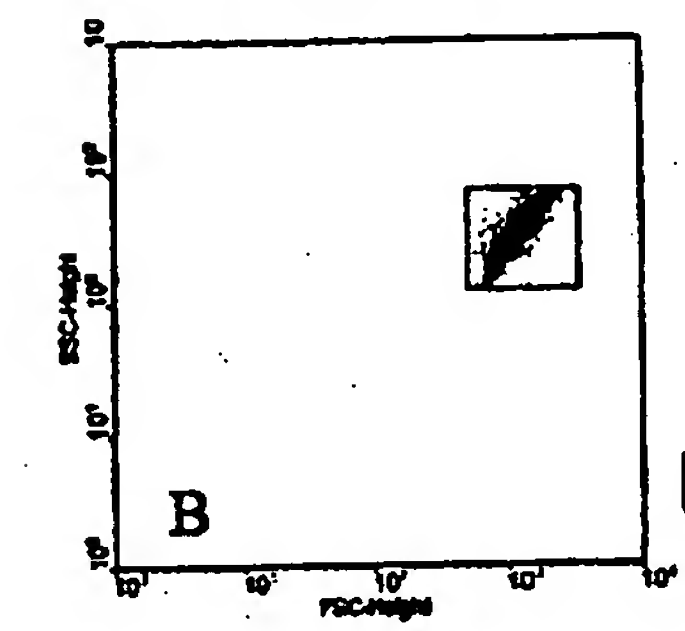

Region 1 = outer region
Region 2 = inner region
SSC-H
FSC-H
FL1-H
A
B
C
D
Rerun
Rerun

Figure 33

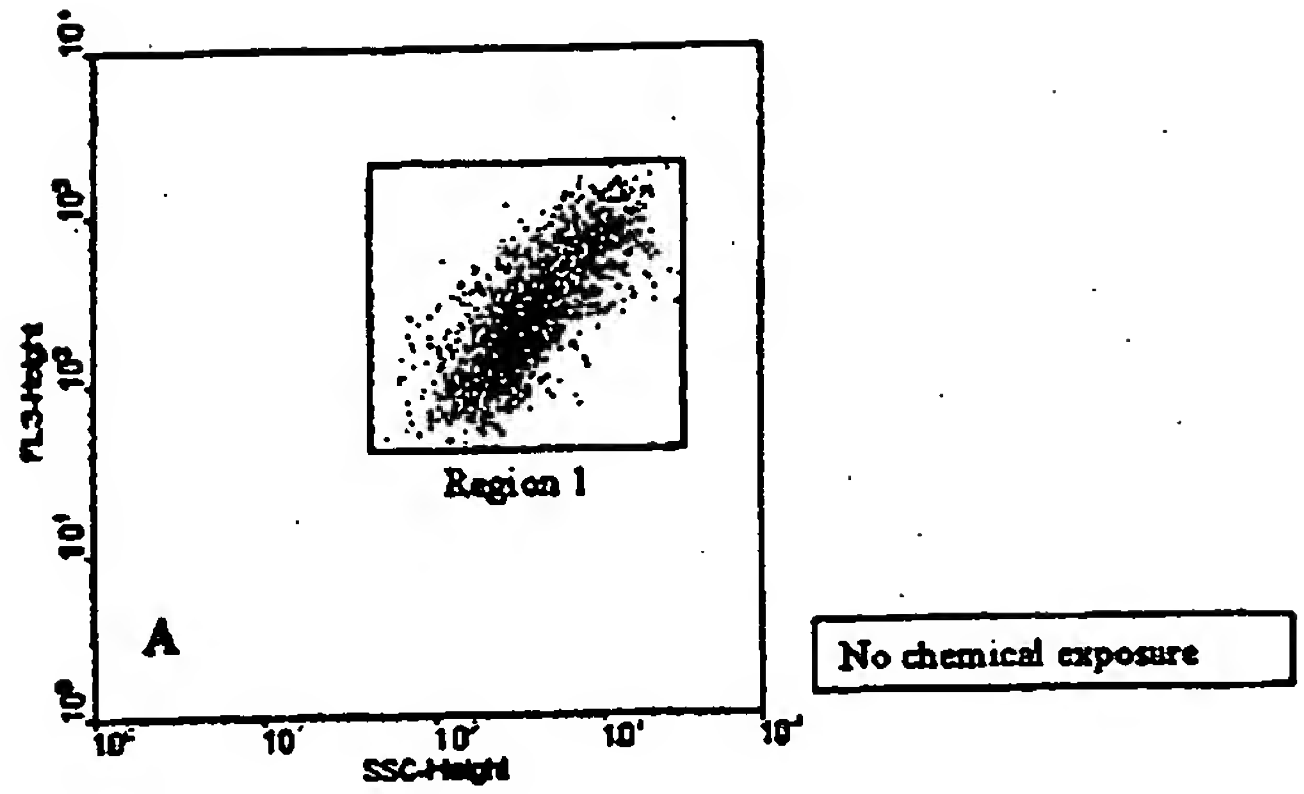


No DMF exposure



DMF exposure for 3 hours

Figure 34


Region 1
A
No chemical exposure
FL3-Height
SSC-Height

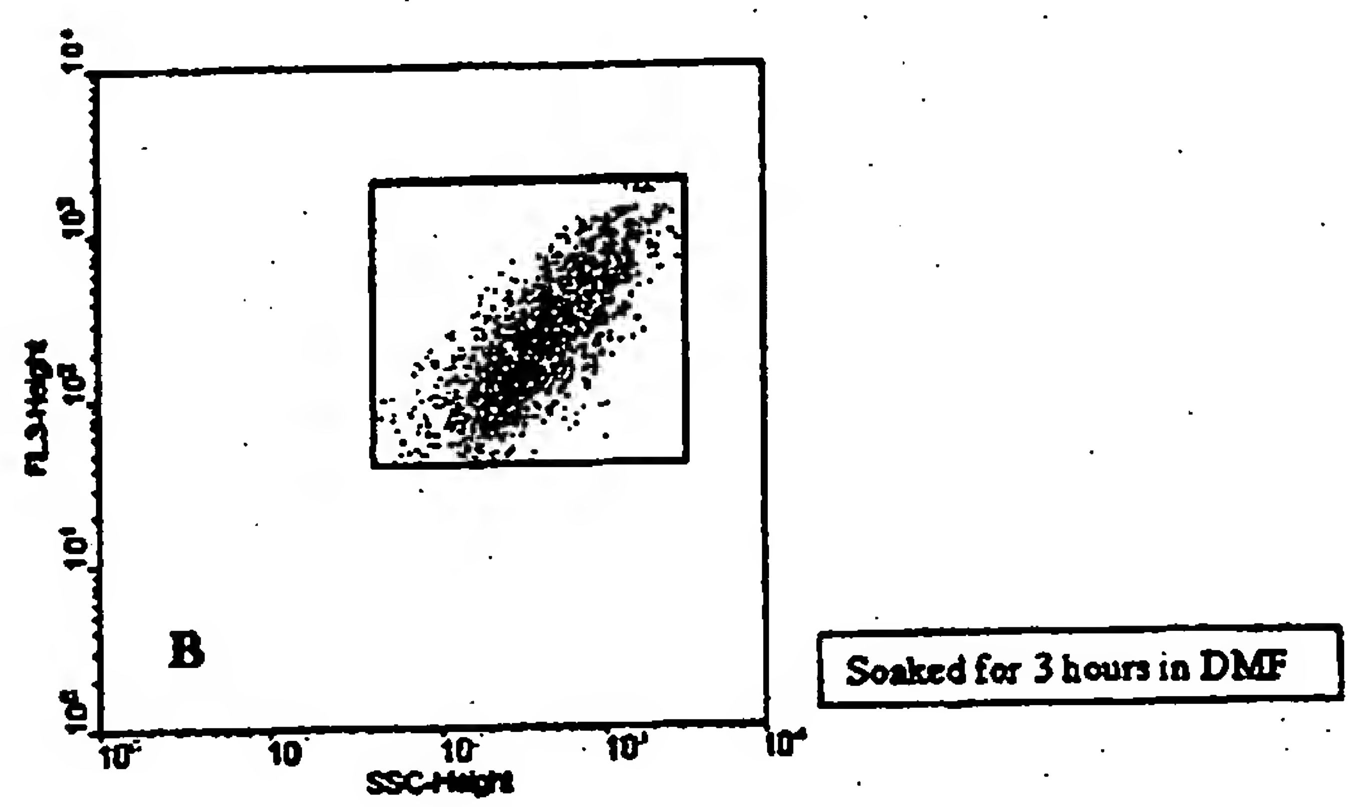
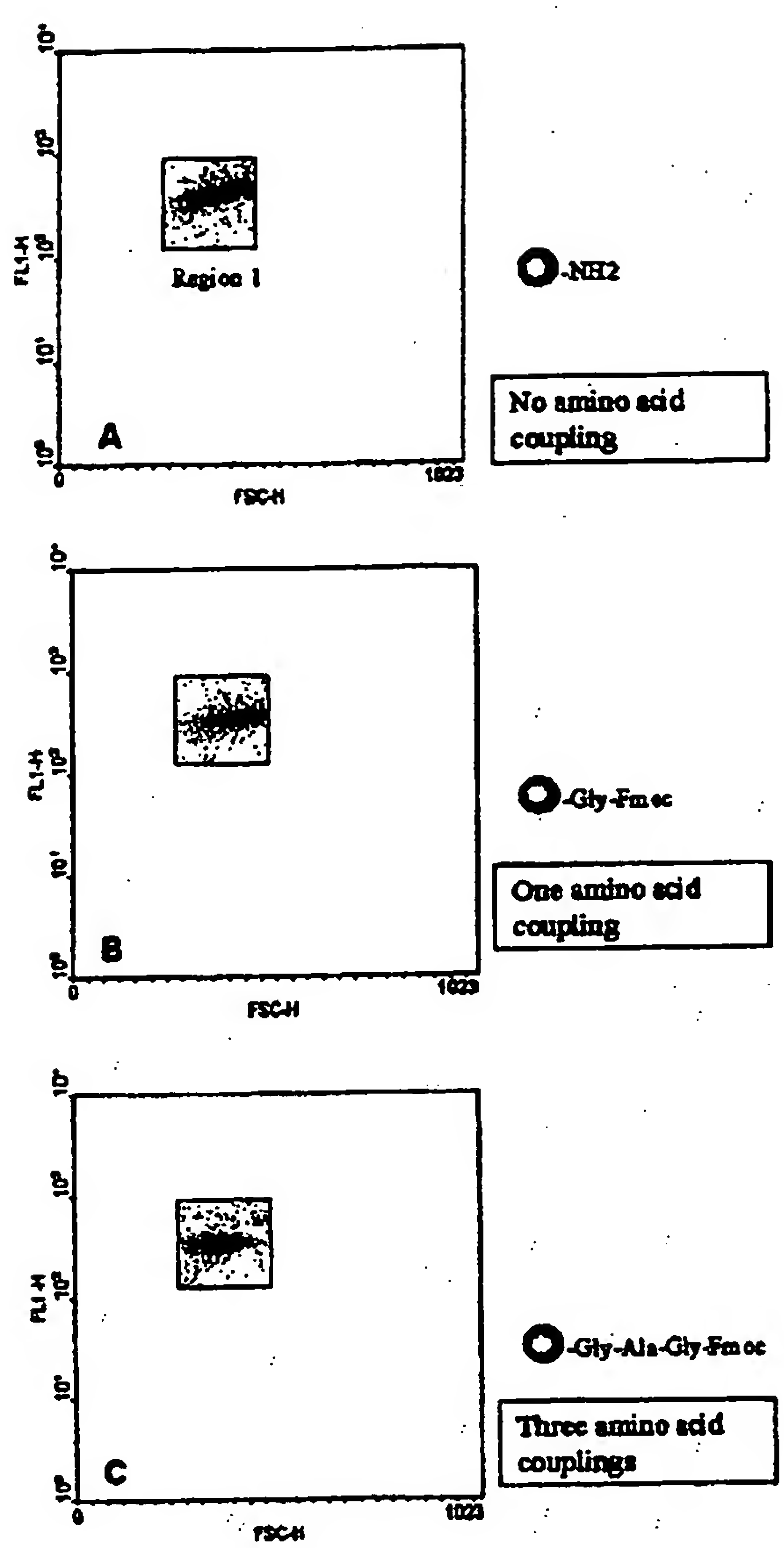

B
Soaked for 3 hours in DMF
FL3-Height
SSC-Height

Figure 35



FL1-H
FSC-H
Region 1
A
-NH2
No amino acid coupling
B
-Gly-Fmoc
One amino acid coupling
C
-Gly-Ala-Gly-Fmoc
Three amino acid couplings

Figure 36

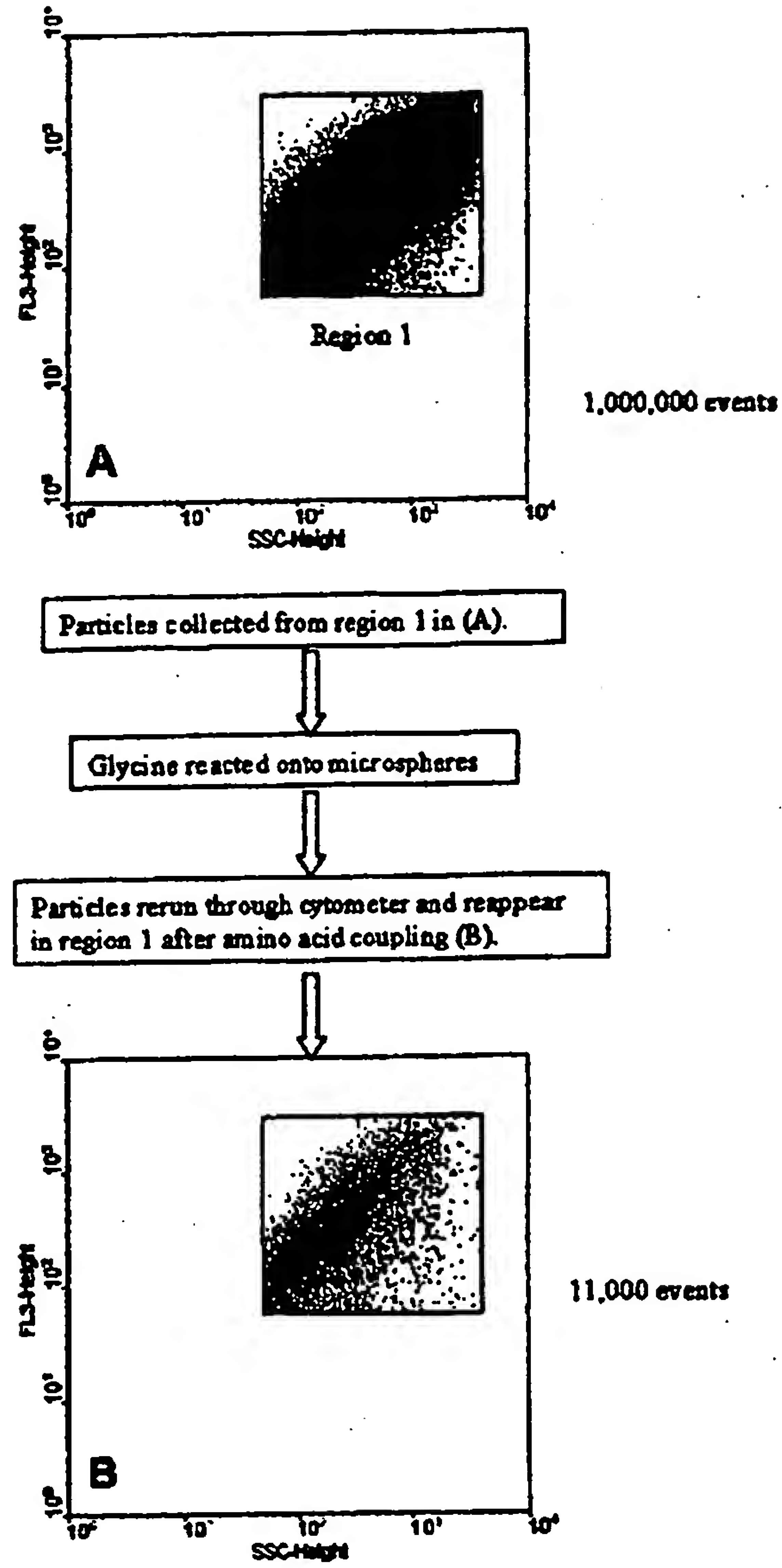

FL3-Height
Region 1
1,000,000 events
A
SSC-Height
Particles collected from region 1 in (A).
Glycine reacted onto microspheres
Particles rerun through cytometer and reappear in region 1 after amino acid coupling (B).
FL3-Height
11,000 events
B
SSC-Height

Figure 37

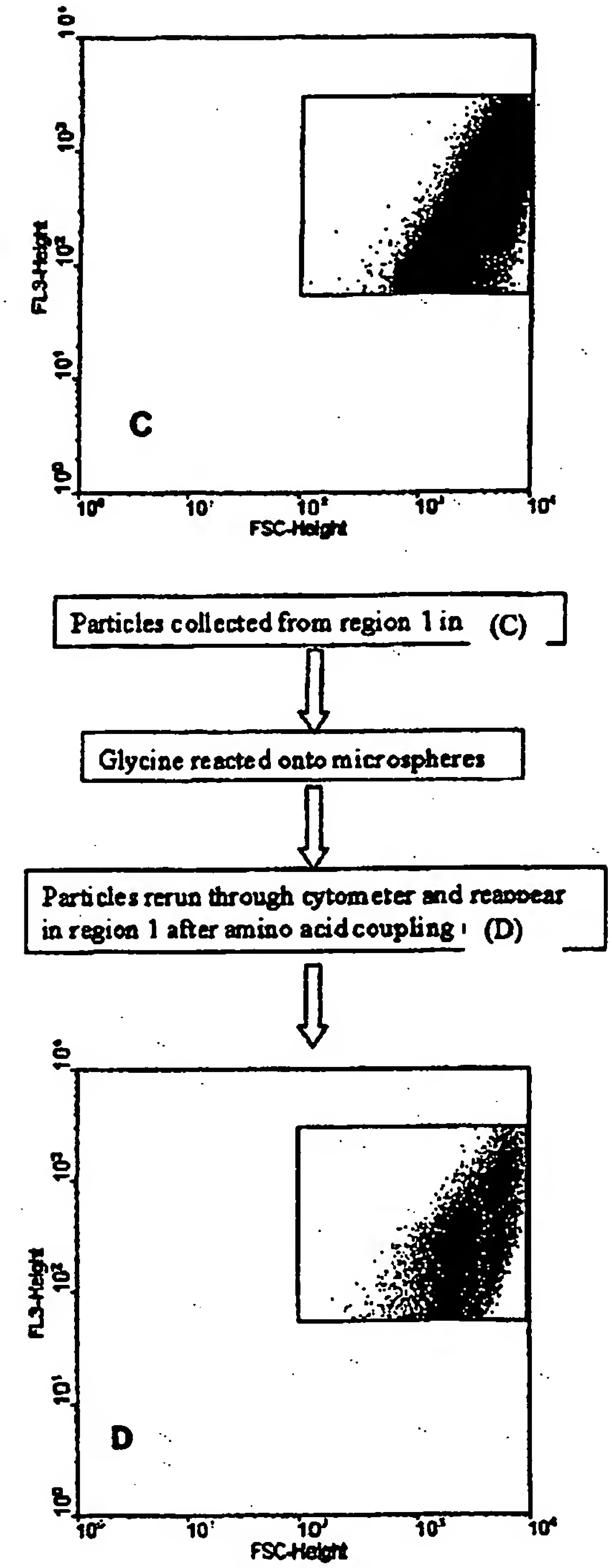


Figure 37 cont'd

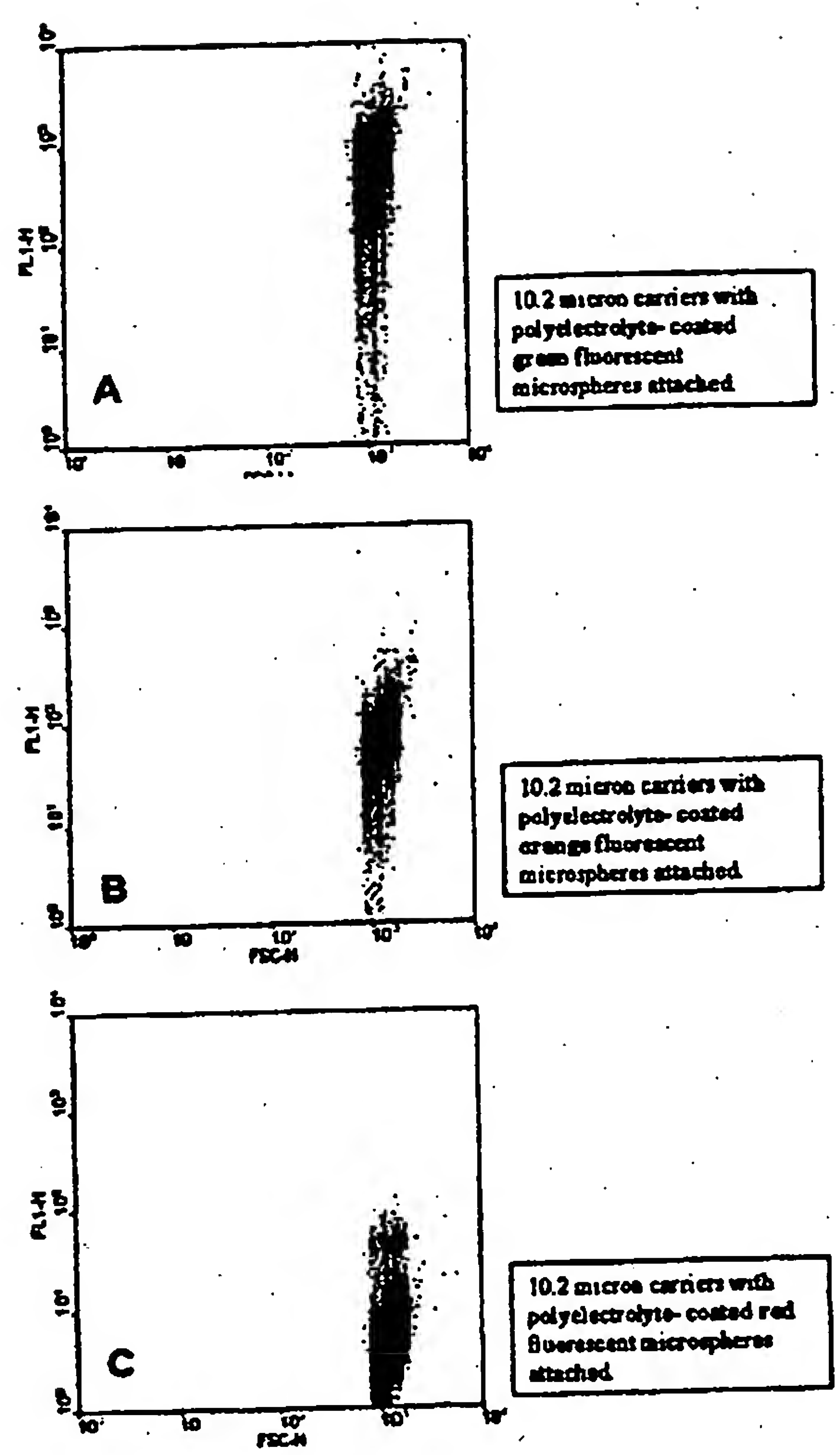

FL1-H
FSC-H
A
10.2 micron carriers with polyelectrolyte- coated green fluorescent microspheres attached
B
10.2 micron carriers with polyelectrolyte- coated orange fluorescent microspheres attached
C
10.2 micron carriers with polyelectrolyte- coated red fluorescent microspheres attached

Figure 38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/01065

A.	CLASSIFICATION OF SUBJECT MATTER
Int Cl[6]:	C07B 61/00 //C09B 69/10, 11/08, C07H 21/02, 21/04, 1/00, C07K 17/08, 17/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN 'CA, MEDLINE, WPIDS' Files. (#Keywords: Cytometers, FACS, Combinatorial, carrier:,solid:,support:)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	Derwent Abstract Accession No. 96-020681/02, Class B04, WO 95/32425 A (SMITHKLINE BEECHAM CORP.) 30 November 1995 See whole abstract, especially the 'Example'.	1, 15, 30, 48
X,Y	STN Medline On-line Abstract Accession No. 94285715, & Nogueira A.C. et.al., LIFE SCIENCES (1994) 55(2) 77-92. See whole abstract, especially, "beads bearing a determined number of fluorescence molecules".	1, 15, 30, 48
Y	STN Medline On-line Abstract Accession No. 94068469, & Needels M. C. et.al., PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1993 Nov 15) 90(22) 10700-4. See whole abstract.	1-39

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 1999	06 JAN 2000

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/01065

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AU 11280/95 (703472) B (AFFYMAX TECHNOLOGIES N.V.) 23 May 1995 (&Chemical Abstract 127:119338, "Synthesizing and screening molecular diversity", see whole abstract)	1-39
Y	Chemical Abstract 123:314496, "Applications of encoded synthetic libraries in ligand discovery". & Polym. Prepr.(Am.Chem.Soc., Div. Polym. Chem) (1994),35(2). 981-2. See whole abstract.	1-39

INTERNATIONAL SEARCH REPORT	International application No. PCT/AU 99/01065

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **1-38**
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

the claims are very broad in scope and a complete search is not feasible for economic reasons. The search was limited to the carriers exemplified in the specification.

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/01065

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	11280/95	BR	9407947	CN	1134156	EP	726906
		EP	773227	GB	2298863	JP	09508353
		US	5639603	US	5770358	WO	9512608
WO	9532425	EP	763202	JP	10500951		

END OF ANNEX